CHAPTER 2

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

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ABSTRACT

Most protein aggregation-associated neurodegenerative diseases are associated with activation of astrocytes. Astrocytes are activated in early stages of these diseases, however, their contribution to pathogenesis is unclear. Cellular stress or damage in neurons (cell-autonomous contribution) is associated with neurodegeneration. In addition, cell non-autonomous contributions of non-neuronal cells may also contribute to neurodegeneration.

Here, we established a *Drosophila* (fruit fly) model to analyze whether astrocytes can contribute to neurodegeneration in a cell non-autonomous manner. In a candidate RNAi screen targeting astrocytes in a fly model for neurodegeneration, we identified genes that could non-autonomously affect tissue degeneration. We examined these genes in a *Drosophila* model for Spinocerebellar Ataxia-3 (SCA3, also known as Machado Joseph Disease), a disease caused by expansion of the polyglutamine (polyQ) stretch in the *ATXN-3* gene. In this model, a biologically relevant, truncated part of the *ATXN-3* gene containing an expanded polyQ stretch (*SCA3*^{polyQ78}) was expressed in cells in eyes, including photoreceptors but excluding astrocytes. Simultaneously, candidate genes were exclusively downregulated in astrocytes. We identified both enhancers and suppressors of SCA3^{polyQ78}-induced eye degeneration, strongly demonstrating that astrocytic functioning can contribute to neurodegeneration.

Our data point to novel mechanisms of cell non-autonomous contributions to neurodegeneration via astrocytes. We speculate about mechanistic contributions of several candidate genes.

INTRODUCTION

Glia are non-neuronal cells in the central nervous system (CNS) and astrocytes form a sub-class of glial cells. In mammals, among all types of cells in the CNS, astrocytes are the most abundant. They are present in the entire CNS and envelop synapses, and are involved in maintaining neurotransmitter homeostasis, synaptic function, energy metabolism and inflammation in the CNS[1]. A number of studies point out that astrocyte dysfunction can cause damage to neurons and contribute to disease development, such as stroke and epilepsy[2]. Also, neurodegenerative diseases (NDs) are associated with changes in activation of astrocytes[2].

A feature of most age-related NDs, such as Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic Lateral Sclerosis (ALS), Huntington's disease (HD), and different types of Spinocerebellar ataxias (SCAs) is the accumulation of misfolded or aggregated proteins[3]. Neuronal loss in NDs is associated with neuronal accumulation of toxic misfolded proteins. These neuronal misfolded proteins contribute to neuronal death or damage in a cell-autonomous manner. However, cell non-autonomous contributions from non-neuronal cells, such as astrocytes, may also contribute to neuronal damage or influence neuronal functioning. In reactive astrocytes that are associated with NDs or neuronal damage, the physiological functions can be altered, which consequently could lead to a further increase in neuronal damage (cell non-autonomous contribution). Astrocytes are activated in most if not all NDs[4]. However, the signals that mediated activation of astrocytes, as well as which signaling events in astrocytes may modulate neuronal functioning in neurodegeneration remain to be identified.

Astrocytes can respond to a variety of signaling molecules that are released from other cells. These signaling molecules are amongst others DAMPs (danger or damage-associated molecular patterns), which are released from damaged or dying cells or can be cytokines or neurotransmitters[5]. Receptors for the DAMPs are PRRs (pattern recognition receptors), which are expressed on astrocytes and binding of DAMPs to PRRs in astrocytes could contribute to their activation (reviewed in[6]). Astrocytes can be stimulated by mitochondrial DNA (a DAMP) to produce pro-inflammatory cytokines (reviewed in[7]). In NDs, misfolded or aggregated proteins, can be released from the damaged or dying neurons and can also act as DAMPs and stimulate astrocytes. It has indeed been shown that clearance of aggregates mediated by astrocytes can occur in NDs, however the mechanisms behind this are not known[8]. Here we will focus on the signaling in astrocytes that is triggered by neurons that express aggregation-prone proteins. Misfolded or aggregated proteins function as ligands in one class of PRRs, Toll-like receptors (TLRs)[9]. Upon binding of the ligand to the TLR, intracellular signaling cascades are initiated,

leading to increased pro-inflammatory cytokines synthesis. However, it remains to be determined whether PRRs in astrocytes contribute to neurodegeneration. Besides PRRs, stimulation of pro-inflammatory cytokine receptors on astrocytes also contributes to their activation. Interleukin 1 beta (IL-1β) can stimulate astrocytes to produce the pro-inflammatory cytokines interleukin 6 (IL-6)[10] and TNFα[11]. Thus, increased pro-inflammatory cytokine production in neurodegenerative diseases can also lead to activation of astrocytes, resulting in further cytokine production[12].

In a healthy individual, astrocytes are maintained in a quiescent state. In NDs, this quiescent state is disrupted by alterations in signals (reviewed in[6]). In general, the receptors that are engaged by astrocytes to become activated and to contribute to NDs, such as individual PPRs and cytokine receptors, remain to be identified. Other transmembrane proteins present at the plasma membrane of astrocytes may play a role in their activation as well. A previous study showed that knocking out integrin subunit β1 specifically in astrocytes in a mouse model resulted in activation of astrocytes[13]. This suggests that integrins are necessary to keep astrocytes in a resting state. Integrins are transmembrane proteins, which consist of an α subunit and a β subunit heterodimer. They are involved in cell adhesion and signaling between cells as well as in cell migration. However, it is unclear how alterations in integrin signaling can result in the activation of astrocytes.

In NDs, there are a number of functional changes in astrocytes, illustrated by the observation that the capacity of maintaining neurotransmitter homeostasis in astrocytes can be altered in NDs[14]. Altered homeostasis of neurotransmitters is harmful to neurons[14] and contributes to neurotoxicity. Levels of the excitatory neurotransmitters, such as glutamate, are elevated and are toxic to neurons[15]. In a healthy individual, astrocytes efficiently take up the extracellular glutamate by glutamate transporters, known as excitatory amino acid transporters (EAATs)[16]. In NDs, activated astrocytes are less efficient in clearing excessive extracellular glutamate, which consequently causes neuronal damage[17]. Not only the homeostasis of excitatory neurotransmitters but also of inhibitory neurotransmitters, such as gamma-aminobutyric acid (GABA), is altered in astrocytes[18,19]. Changes in both uptake, as well as in release of GABA in reactive astrocytes have been reported[4]. In AD patients as well as in an AD mouse model, astrocytes have elevated intracellular levels of GABA[18]. This suggests that changes in GABA homeostasis in astrocytes may be associated with NDs. In an AD mouse model, reactive astrocytes release excessive levels of GABA, which contribute to impaired learning ability and memory[20]. These defects are fully restored upon suppression of GABA synthesis or release in astrocytes. However, the molecular mechanisms of GABA homeostasis regulation in astrocytes have not been fully elucidated yet.

Besides changes in the regulation of neurotransmitter levels, calcium homeostasis in astrocytes is also altered in NDs. Alterations in calcium homeostasis in astrocytes will affect calcium-dependent intracellular signalling[1]. Enhanced calcium-induced signaling in astrocytes has been observed in AD[21] as well as in ALS[22] models. Calcium can activate downstream signaling via the calcium/calmodulin-dependent serine-threonine phosphatase, calcineurin (reviewed in[23]). This results in the activation of calcineurin-dependent transcription factor, NFAT (Nuclear Factor of Activated T cells)[23]. The importance of calcineurin signaling in activation of astrocytes has been demonstrated (reviewed in[24]). Calcineurin activity is upregulated in aging and AD models[25]. Moreover, there is an NFAT binding site in the promoter of glutamate transporter (EAAT2)[26], although the direct regulation has not been studied yet. This may indicate a potential regulation of calcineurin and glutamate homeostasis in astrocytes.

PRRs and cytokine receptors can promote intracellular signaling to activate the transcription factor NF-κB to produce pro-inflammatory cytokines. In NDs, the NF-κB is activated in astrocytes[4], suggesting that this transcription factor may contribute as well. However, which molecules in astrocytes are important for the regulation of NF-κB signaling have not been fully elucidated. To what extent NF-κB, calcineurin signaling, neurotransmitter homeostasis, and calcium homeostasis contribute to NDs is also not well understood.

Intracellular signaling in astrocytes results in the secretion of molecules that are secreted by astrocytes to signal to neurons. For instance, altered calcium homeostasis in astrocytes results in changes in the release of gliotransmitters, such as glutamate, secreted by glia required for glia-neuron communication[27]. Dysregulation of gliotransmitter secretion can cause neuronal damage. For instance, excessive levels of glutamate were released from astrocytes in a calcium-dependent manner when astrocytes were exposed to amyloid beta peptides, resulting in synaptic damage[28]. Furthermore, activation of calcineurin results in astrocytic inflammatory responses, through which the secreted neurotoxic factors can also cause neuronal damage. Therefore, it is important to understand which molecules are involved in releasing signals from astrocytes to neurons in NDs.

Thus, in NDs, astrocytes can be activated as a result of neuronal signaling. Consequently, the activated astrocytes can signal to neurons. Currently, it is unclear how cell non-autonomous signaling from astrocytes to neurons contributes to NDs. Earlier work has demonstrated that expression of aggregation-prone proteins in astrocytes can cell non-autonomously influence neuronal viability[29] (reviewed in[6]). However, it is unclear whether signaling in astrocytes can influence neuronal viability when aggregation-prone proteins are expressed specifically in neurons.

We investigated if and how astrocytes contribute to neurodegenerative diseases in a cell non-autonomous manner, within the context of an intact animal. Examining astrocytes in an *in vivo* model is key, given that their morphology and activity changes when taken outside their physiological context (reviewed in[30]). For this, we conducted a dedicated RNAi screen to selectively knock down individual genes in astrocytes in a *Drosophila melanogaster* (fruit fly) model of the polyQ disease SCA3. In SCA3, the *ATXN-3* gene contains an expanded CAG repeat (coding for glutamine) that leads to the expression of a misfolded aggregation-prone ATXN-3 polyglutamine protein. These misfolded polyQ-containing proteins accumulate intracellularly, resulting in neuronal damage and activation of astrocytes (reviewed in[31]). In SCA3, the stretch of polyglutamine repeats is in the range of 62 to 86 glutamines[32]. Activated astrocytes were found in SCA3 patients[33], suggesting potential contributions of astrocytes in the pathogenesis of SCA3.

To independently manipulate neurons and astrocytes, *Drosophila melanogaster* is a suitable model organism. *Drosophila* has been successfully used as an organism for genetic screens for over a century, which has yielded fundamental insights in biology and in human health. More than half of the *Drosophila* genes have orthologs in human, and nearly 75% of disease-associated genes in humans have orthologs in *Drosophila*[34]. Moreover, many physiological processes are conserved from fly to human. To gain insight into human diseases using *Drosophila*, either the ortholog of the disease-causing gene can be mutated in *Drosophila*, or alternatively, a human disease-causing gene can be expressed in *Drosophila*. Expression of human amyloid beta peptides, associated with Alzheimer's disease, causes neurodegeneration and shortening of lifespan in *Drosophila*[35]. Similarly, expression of a biologically relevant part of the *ATXN-3* gene, containing an expanded polyQ stretch, SCA3^{polyQ78}, associated with SCA3, resulted in neurodegeneration[36]. As a model for neurodegeneration, the *Drosophila* eye was used in this study: eye-specific expression of genes associated with neurodegeneration can also cause eye degeneration. Expression of SCA3^{polyQ78} in the *Drosophila* eye results in an easily screenable phenotype[36]. An advantage of this approach is that the eye can easily and quickly be screened, and does not require time-consuming procedures such as analysis of lifespan. To assess the relevance of cell non-autonomous contributions of astrocytes to a neurodegenerative disease associated with aggregation, SCA3, we expressed SCA3^{polyQ78} specifically in *Drosophila* eyes and simultaneously downregulated expression of candidate genes exclusively in astrocytes. The availability of fly lines that express RNAi constructs and genetic tools allow specific down-regulation of candidate genes in astrocytes. We carried out a candidate RNAi screen of genes that are putatively involved in recognizing signals from neurons (receptors), intracellular signaling or genes that encode putative signaling molecules that can signal to neurons (such as neuropeptides).

Similar to mammals, astrocytes are important for neuronal functioning in *Drosophila*[31]. *Drosophila* astrocytes share structural similarities with mammalian astrocytes, such as a branched appearance[37]. The distribution of *Drosophila* astrocytes is also comparable with mammalian astrocytes, as they connect with the blood-brain barrier and fill in the spaces between neurons[37]. Similar to mammalian astrocytes, they play an important role in sensing as well as clearing of glutamate (reviewed in[31]). Some conserved genes in *Drosophila* astrocytes have been identified. For example, glutamate transporters (EAATs) are expressed in *Drosophila* astrocytes. *Drosophila EAAT1* is orthologous to the mammalian EAATs, *GLAST* and *GLT-1*[38]. Similar to vertebrates, there are also inhibitory neurotransmitters in the *Drosophila* CNS, such as GABA. GABA-A receptors have orthologs in *Drosophila*: *ligand-gated chloride channel homolog 3 (Lcch3), Resistant to dieldrin (Rdl)* and *Glycine receptor (Grd)*[39]. *Drosophila* astrocytes express *NF-κB* genes and *calcineurin* genes[40], however, their functions have not been examined in *Drosophila* astrocytes. Some aspects of astrocytic functioning are not conserved. For example, adult astrocytes in *Drosophila* do not contribute to clearance of degenerating neurons[41].

We performed a candidate screen to investigate whether RNAi-mediated downregulation of genes in astrocytes could influence the extent of degeneration in eyes expressing SCA3^{polyQ78}. Identification of enhancers or suppressors will demonstrate cell non-autonomous involvement of astrocytes and shed light on the relevant signaling molecules in astrocytes. This setup allows screening of a large number of genes (around 160) in a short time frame.

We analyzed putative involvement of genes in astrocytes in the recognition of signals from SCA3^{poly78}-expressing eyes, intracellular signaling and genes involved in generation of signals from astrocytes (gliotransmitters or neuropeptides) that could influence the extent of SCA3^{poly78}-induced degeneration. Analysis of genes may provide answers to the following questions:

1. What are the signals from degenerating neurons that signal to astrocytes?
2. Which intracellular signaling pathways in astrocytes contribute to polyQ disease?
3. What are the signaling molecules released by astrocytes that influence neurodegeneration?

RESULTS AND DISCUSSION

Generating SCA3^{polyQ27} and SCA3^{polyQ78} in the Q system

Previous studies have shown that *Drosophila* eyes are a suitable model to study SCA3[36,42]. Expressing a biologically relevant, truncated fragment of the SCA3 disease-causing protein of the human *ATXN-3* containing the expanded polyglutamine stretch, SCA3^{polyQ78}, specifically in *Drosophila* eyes, resulted in a degenerative eye phenotype[43]. This distinct phenotype is easily screenable for modifiers. Similar to ATXN-3 containing an expanded polyQ stretch in SCA3 in humans, SCA3^{polyQ78} in *Drosophila* forms aggregates. The degenerative eye phenotype, as well as the extent of SCA3^{polyQ78} aggregation, can be used to screen for modifiers and thus identify genes that are relevant in SCA3. Such screens have been successfully done and yielded novel insight into SCA3[42]. However, these screens were performed to identify cell-autonomous modifiers of SCA3, identifying genes that are also expressed in the same cells of the eye as SCA3^{polyQ78} protein.

Tissue-specific gene expression in *Drosophila* has been established by using a binary expression system that was derived from yeast, UAS-GAL4[44]. The UAS-GAL4 consists of two components: the GAL4 transcription factor and the UAS promoter. GAL4 binds to the UAS promoter to activate the expression of genes under the control of GAL4-specific UAS (upstream activating sequence). Tissue-specific expression of GAL4 in *Drosophila* has no effect, and a gene under control of the UAS promoter (*UAS-gene*) is not expressed in the absence of GAL4. However, the combined presence of both GAL4 and *UAS-gene* results in expression of the gene in the tissues that express GAL4. One advantage is that a gene that would be toxic when ubiquitously expressed can be analyzed in a tissue that is not essential for *Drosophila* viability, such as eyes or wings.

To specifically express SCA3^{polyQ78} in the *Drosophila* eye, we used the Q system. The Q system also consists of two components: the transcription factor QF2 and the QUAS sequence, which is the promoter sequence for QF2. QF2 activates the expression of genes under the control of QUAS. This system has recently been employed in *Drosophila* and its functioning independent of UAS-GAL4 has been established[45]. We used the Q system to express human SCA3^{polyQ78} in *Drosophila* eyes and the UAS-GAL4 system to express RNAi constructs in astrocytes.

We express SCA3^{polyQ78} in the *Drosophila* eye and analyze whether astrocytes can contribute to the degenerative phenotype. We analyzed the involvement of specific genes in astrocytes in SCA3^{polyQ78}-induced eye degeneration. For this, we downregulated expression of individual genes in astrocytes, using RNAi constructs.

Thus, to enable simultaneous modulation of gene expression (expression of SCA3^{polyQ78} in eyes and RNAi constructs in astrocytes), we combined the Q system (to express SCA3^{polyQ78} specifically in eyes) with the UAS-GAL4 system to modulate gene expression in astrocytes.

We used a fly line that expressed QF2 under the control of an eye-specific promoter GMR, resulting in a QUAS-dependent expression that was exclusively restricted to the eye (Figure 1a). As the Q system has not been used before to express human *ATXN3* in *Drosophila* tissue, we first compared expression levels of a truncated fragment of human *ATXN3* containing either a non-pathogenic glutamine stretch of 27 glutamines (SCA3^{polyQ27}) or a pathogenic length of 78 glutamine repeats

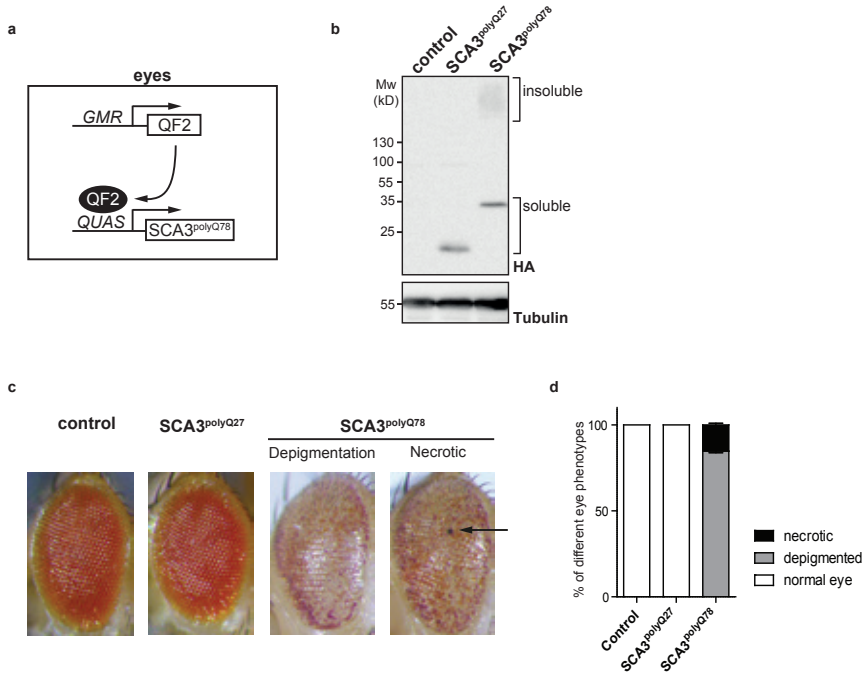

Figure 1. The Q system was used to express truncated human *ATXN-3* (*SCA3*) protein containing different lengths of polyglutamine (polyQ) repeats in *Drosophila* eyes.
(a) To express human *ATXN-3* in *Drosophila* eyes we used the Q system, using an eye-specific QF2, *GMR-QF2* to express *QUAS-SCA3^{polyQ27}* or *QUAS-SCA3^{polyQ78}*.
(b) The expression levels and extent of aggregation of HA-tagged SCA3^{polyQ27} and SCA3^{polyQ78} were analyzed on Western blot. Tubulin was used as a loading control. Figures represent two-time experiments.
(c) Eye phenotypes of SCA3^{polyQ27} or SCA3^{polyQ78} expression. SCA3^{polyQ78}-induced phenotypes are depigmentation and necrotic spots ('necrotic'). The arrow points at a necrotic spot. Figures represent at least three experiments.
(d) Quantification of the eyes that have a normal appearance, display depigmentation or necrotic spots as shown in (c). n=3.
Genotypes in (b), (c) and (d): control, *GMR-QF2/+*. SCA3^{polyQ27}, *GMR-QF2/+; QUAS-SCA3^{polyQ27}/+*. SCA3^{polyQ78}, *GMR-QF2/+; QUAS-SCA3^{polyQ78}/+*.

(SCA3^{polyQ78}). Also, we tested whether expression of SCA3^{polyQ78} (but not SCA3^{polyQ27}) could induce a degenerative eye phenotype.

We checked expression levels and extent of aggregation of SCA3^{polyQ27} and SCA3^{polyQ78} by analyzing head lysates of flies expressing SCA3 in the eyes on Western blot. Expression levels of SCA3^{polyQ27} and SCA3^{polyQ78} were comparable (Figure 1b), but only expression of SCA3^{polyQ78} resulted in the formation of insoluble aggregates of SCA3^{polyQ78} (Figure 1b). Expression of SCA3^{polyQ27} in *Drosophila* eyes did not induce eye degeneration (Figure 1c), whereas expression of pathogenic SCA3^{polyQ78} in *Drosophila* eyes resulted in degeneration, as shown by loss of pigmentation (Depigmentation, mild degeneration) or loss of pigmentation together with the presence of necrotic patches (Necrotic, severe degeneration) (Figure 1c, arrow). When we expressed SCA3^{polyQ78} in *Drosophila* eyes, about 20% of the eyes displayed severe degeneration while the rest of the eyes were mildly degenerated (Figure 1d). No degeneration was observed in control eyes or in eyes expressing SCA3^{polyQ27}. We used the fraction of eyes displaying necrotic spots to quantify the extent of degeneration. The observation that SCA3^{polyQ78} formed aggregates and induced degeneration whereas no degeneration or aggregation was seen with SCA3^{polyQ27}, demonstrates that protein misfolding or aggregation accounts for the degeneration, as shown before[42].

Thus, the Q system we used to express SCA3^{polyQ78} can be used as a tool to study and quantify misfolded protein-associated degeneration.

Simultaneous and independent modulation of gene expression in eyes and astrocytes

Combining two binary expression systems allows independent manipulation of gene expression in different tissues. To study the effect of astrocytes on SCA3^{polyQ78}-induced eye degeneration, we used the Q system to express SCA3^{polyQ78}, and the UAS-GAL4 system to modulate gene expression in astrocytes. In this study, we used GAL4 expressed under control of the astrocyte-specific promoter *alrm*[41] to specifically modulate expression of UAS constructs in astrocytes. We visualized localization of astrocytes in the *Drosophila* brain by combining *alrm-GAL4* with *UAS-RFP* allowing the expression of *RFP* in astrocytes. As shown before[46], in the adult fly brain astrocytes are present in the lamina and medulla region of the brain where the axons of photoreceptors terminate (Figure 2). The lamina region is a superficial layer and the medulla region is the deeper layer[47]. This localization indicates possible interactions between the photoreceptors and astrocytes[48]. This also may indicate that astrocytes contribute to the functioning of photoreceptors, a hypothesis that can be further tested in the presented screen.

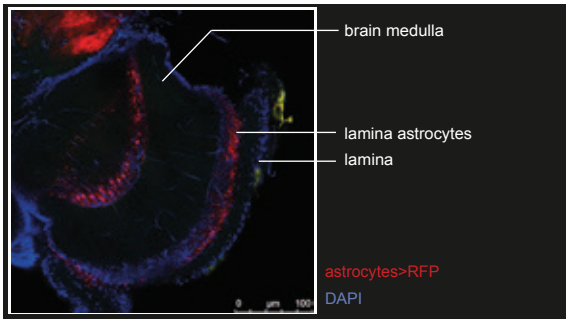

Figure 2. The localization of astrocytes in the fly brain. The representative figure to show the localization of astrocytes in lamina and medulla in the adult fly brain was visualized by expressing *RFP* specifically in astrocytes (red), using the UAS-GAL4 system. Nuclei were stained with DAPI (blue). Genotype: *alrm-GAL4/ UAS-RFP*.

A screen to elucidate possible cell non-autonomous roles of astrocytes in SCA3

To investigate whether genes in astrocytes can cell non-autonomously contribute to SCA3^{polyQ78}-induced degeneration, a candidate RNAi screen in astrocytes was carried out in the SCA3^{polyQ78} eye model.

As Figure 3a shows, the Q system was used to express *SCA3^{polyQ78}* in *Drosophila* eyes, and the UAS-GAL4 system was used to knock down individual genes specifically in astrocytes by expressing different *UAS-RNAi* constructs. This way, we can independently manipulate gene expression in the eye and in astrocytes. Indeed, in pupae, expression of RFP (via UAS-GAL4) in astrocytes and GFP (via the Q system) in the developing eyes shows that there is no overlap in expression (Figure 3b). In our candidate RNAi screen, RNAi constructs targeting a set of 156 selected genes were expressed exclusively in astrocytes in flies expressing SCA3^{polyQ78} exclusively in the eyes, and the extent of eye degeneration was analyzed. We quantified and compared the extent of degeneration (necrotic phenotype, Figure 1c) in fly eyes expressing SCA3^{polyQ78} to fly eyes expressing SCA3^{polyQ78} together with an RNAi construct in astrocytes. We selected genes (Table 1) potentially involved in (1) receiving signals from neurons, such as DAMPs, neuropeptides and neurotransmitters. (2) intracellular signalling pathways in astrocytes, such as immune signaling pathways, nuclear factor kappa B (NF-κB) and (3) signaling of molecules that might be released by astrocytes.

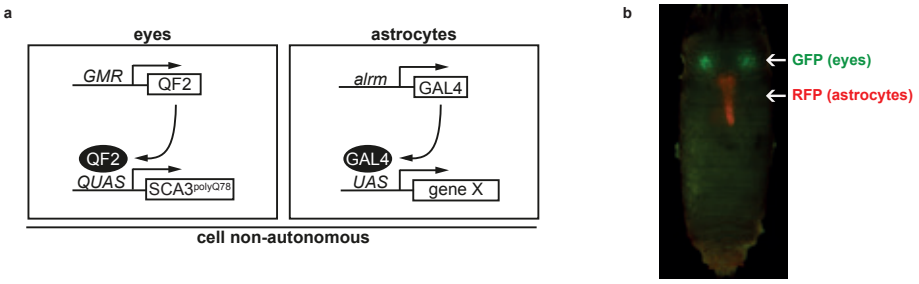

Figure 3. Genetic setup of candidate RNAi screen to study the cell non-autonomous roles of astrocytes in SCA3.

(a) Two binary expression systems were combined in the screen to allow independent regulation of expression in eyes and astrocytes. The Q system was used to express *SCA3*^{polyQ78} in fly eyes. To knock down individual genes in astrocytes, the UAS-GAL4 system was used. Different *UAS-RNAi* constructs were expressed specifically in astrocytes using *alrm-GAL4*.

(b) Independent expression of the QUAS-QF system (green, eyes) and the UAS-GAL4 system (red, astrocytes) in the late pupa. Genotype, *GMR-QF2/+; alrm-GAL4/UAS-RFP; QUAS-mCD8-GFP/+*. The figure is representative of two independent experiments.

A gene was identified as an enhancer gene of SCA3^{polyQ78}-induced degeneration, when astrocyte-specific downregulation of this gene resulted in a reduction of more than 40% of the SCA3 ^{polyQ78} severe degenerative eye phenotype. A suppressor gene was identified when its downregulation resulted in an increase of more than 60% of the SCA3^{polyQ78} severe degenerative phenotype. In total, 156 genes were tested in our screen, resulting in the identification of 19 enhancers and 25 suppressors (Table 1). Additional experiments need to be done to confirm the identified enhancers or suppressors. Independent RNAi lines targeting a different part of the target candidate should be used to exclude off-target effects. In addition, the efficiency of the RNAi-mediated knock down needs to be investigated by qPCR to confirm efficient knockdown of the gene. The identification of suppressors and enhancers indicate that astrocytes make cell non-autonomous contributions to the SCA3^{polyQ78} eye phenotype.

To decide which genes would be of interest to further investigate, we grouped the identified enhancer or suppressor genes that have similar functions or belong to the same signaling pathway in astrocytes. One example is a group of genes that belong to an NF-κB (Relish) signaling pathway (Table 1, indicated in green). We showed that the transcription factor Relish acted as an enhancer of SCA3^{polyQ78}-induced degeneration. *PGRP-LE*[49,50], which can activate Relish was also enhancers. Moreover, downregulation of other genes in the Relish pathway (such as *Dredd)* showed similar effects on the SCA3 phenotype. In chapter 3, further experiments

Signaling		
gene		VDRC stock
CG17336, Lcch3	212,48%	109606-KK
CG6378, SPARC	168,48%	100566-KK
CG14358, CCHa1	156,06%	104974-KK
CG8394, VGAT	141,03%	103586-KK
CG18176, defl	117,46%	20604-GD
CG16827, ItgaPS4	109,47%	109783-KK
CG3143, foxo	103,78%	107786-KK
CG3408	101,19%	36306-GD
CG3022, GABA-B-R3	91,30%	108036-KK
CG13758, Pdfr	85,75%	106381-KK
CG6357	82,35%	8782-GD
CG10997, Clic	81,09%	105975-KK
CG7121, Tehao	80,14%	109705-KK
CG7665, Lgr1	77,92%	104877-KK
CG10233, rtp	76,06%	109000-KK
CG3173, IntS1	74,74%	25825-GD
CG5195, atk	74,74%	100110-KK
CG4845, psidin	73,67%	103558-KK
CG5372, ItgaPS5	73,21%	100120-KK
CG14375, CCHa2	73,21%	102257-KK
CG4641,nwk	71,63%	102133-KK
CG17262, cnir	70,19%	104009-KK
CG5528, Toll-9	66,80%	109635-KK
CG8639, Cirl	63,66%	100749-KK
CG8909	62,50%	108629-KK
CG8250, Alk	58,89%	107083-KK
CG9681, PGRP-SB1	58,00%	101298-KK
CG7449, hbs	55,33%	105913-KK
CG1411, CRMP	55,33%	101510-KK
CG43119, Ect4	54,79%	102044-KK
CG11335, lox	53,95%	107435-KK
CG8784, PK2-R1	52,80%	103822-KK
CG4604, Glaz	52,65%	107433-KK
CG7105, Proc	52,00%	102488-KK
CG42611, mgl	50,00%	105071-KK
CG10342, NPF	49,00%	108772-KK
CG31221	48,37%	103017-KK
CG15274, GABA-B-R1	47,15%	105863-KK
CG7250, Toll-6	46,77%	27103-GD
CG2872, AstA-R1	46,70%	101395-KK
CG34399, Nox	46,27%	100753-KK
CG10698, CrzR	45,90%	108506-KK
CG9453, Spn42Da	44,36%	106306-KK
CG13480, LK	44,00%	14091-GD
CG7250, Toll-6	42,69%	928-GD
CG6531, wgn	41,70%	9152-GD
CG18870	40,09%	100135-KK
CG5490, Tl	38,64%	100078-KK
CG11303, TM4SF	36,76%	8847-GD
CG6438, amon	36,00%	110788-KK
CG17800, Dscam1	33,73%	108835-KK
CG7446, Grd	33,14%	5329-GD
CG2736	32,95%	102672-KK
CG6692, Cp1	32,62%	110619-KK
CG8434, Ibk	32,44%	106679-KK
CG31094, LpR1	32,41%	106364-KK
CG32540, CCKLR-17D3	32,05%	102039 KK
CG6456, Mip	31,00%	106076-KK
CG13984	29,91%	101831-KK
CG42613	29,26%	102823-KK
CG5811, Rya-R	26,80%	103973-KK
CG6794, Dif	25,97%	100537-KK
CG7395, sNPF-R	25,90%	9379-GD
CG1147, NPFR	25,40%	9605-GD
CG11217, CanB2	25,08%	104370-KK
CG3131, Duox	24,36%	2593-GD
CG10537, Rdl	24,26%	100429-KK
CG33126, Nlaz	24,26%	101321-KK
CG30340	24,10%	100088-KK
CG34370	22,97%	100162-KK
CG6072, Sra	22,75%	107573-KK
CG4280, crq	22,54%	45883-GD
CG34385, dpr12	21,57%	44741-GD
CG13633, AstA	20,50%	103215-KK
CG33950, trol	20,00%	110494-KK
CG7586, Mcr	19,17%	100197 KK
CG14734, Tk	19,00%	103662-KK
CG4636, SCAR	18,93%	21908-GD

Signaling		
gene		VDRC stock
CG4167, Hsp67Ba	18,74%	104341-KK
CG7000, snmp1	17,00%	104210-KK
CG4096	16,04%	109025-KK
CG13061, Nplp3	16,00%	105584-KK
CG40733, RYa	15,48%	109264-KK
CG7887, TkR99D	15,48%	43329-GD
CG3302, Crz	14,10%	102204-KK
CG4168	13,91%	100080-KK
CG3441, Nplp1	13,00%	14035-GD
CG4432, PGRP-LC	12,80%	101633-KK
CG14593, CCHa2-R	10,74%	100290-KK
CG7228, pes	9,55%	100391-KK
CG31094, LpR1	8,73%	106364-KK
CG4821, teq	8,33%	15362-GD
CG6667, dl	7,23%	45996-GD
CG31619, nolo	6,12%	104736-KK
CG6817, foi	6,05%	10102-GD
CG1804, Kek6	2,52%	109681-KK
CG12079, ND-30	1,67%	103412-KK
CG7285, AstC-R1	1,30%	110739-KK
CG1857, nec	0,17%	108366-KK
CG13419, Burs	-1,00%	111063-KK
CG4437, PGRP-LF	-3,68%	108313-KK
CG3048, Traf4	-4,02%	110766-KK
CG14162, dpr6	-5,38%	103521-KK
CG12004	-5,52%	101732-KK
CG12919, egr	-8,27%	108814-KK
CG13422, GNBP-like3	-8,82%	107358-KK
CG33087, LRP1	-10,78%	109605-KK
CG4545, SerT	-11,71%	11346-GD
CG10823, SIFaR	-11,80%	1783-GD
CG14575, CapaR	-12,41%	105556-KK
CG9623, if	-13,39%	100770-KK
CG1618, comt	-13,80%	105552-KK
CG7285, AstC-R1	-14,41%	13560-GD
CG8942, NimC1	-15,18%	105799-KK
CG5008, GNBP3	-17,65%	37256-GD
CG12489, dnr1	-20,49%	106453-KK
CG9918, PK1-R	-20,75%	101115-KK
CG33717, PGRP-LD	-21,00%	51023-GD
CG10590, TM9SF3	-21,85%	110679-KK
CG8795, PK2-R2	-23,10%	100927-KK
CG7486, Dredd	-23,20%	104726-KK
CG7052, Tep2	-23,67%	106997-KK
CG1632	-24,42%	106107-KK
CG6134, spz	-24,69%	105017-KK
CG8743, Trpml	-25,44%	108088-KK
CG6440, Ms	-27,00%	108760-KK
CG15520, capa	-28,00%	41124-GD
CG11372, galectin	-30,71%	107054-KK
CG11992, Rel	-30,75%	49414-GD
CG14746, PGRP-SC1a	-33,00%	43201-GD
CG6890, Tollo	-33,90%	27099-GD
CG14919, AstC	-34,00%	102735-KK
CG1358	-34,35%	101453-KK
CG6515,TkR86C	-37,64%	107090-KK
CG33696, CNMaR	-40,00%	101076-KK
CG7509	-40,55%	51584-GD
CG30106, CCHa1-R	-40,56%	103055-KK
CG9819, CanA-14F #2	-41,69%	
CG30040, jeb	-43,48%	103047-KK
CG43119, Ect4	-44,57%	105369-KK
CG14928, SPZ-4	-45,35%	7679-GD
CG8329	-45,83%	101603-KK
CG8896, 18w	-46,49%	963-GD
CG31092, LpR2	-48,30%	107597-KK
CG6890, Tollo	-49,84%	9431-GD
CG1771, mew	-50,51%	109608-KK
CG8995, PGRP-LE	-51,14%	108199-KK
CG2086, drpr	-51,37%	4833-GD
CG11051, Nplp2	-54,00%	15305-GD
CG11992, Rel	-56,70%	49413-GD
CG11709, PGRP-SA	-57,00%	5594-GD
CG6706, GABA-B-R2	-63,53%	1785-GD
CG42610, Fhos	-63,53%	34035-GD
CG4099, Sr-CI		110014-KK
CG1732, Gat		106638-KK
CG6378, SPARC		16678-GD

220%	suppressors
180%	
120%	
60%	
20%	
0%	
-20%	
-40%	enhancers
-100%	
	lethal

	Relish pathway genes
	Toll pathway genes

Table 1. The results of the candidate RNAi screen to identify genes in astrocytes that contribute to SCA3^{polyQ78}-induced eye degeneration. The effect of downregulating specific genes in astrocytes on SCA3^{polyQ78}-induced eye degeneration is shown for each gene. The extent of eye degeneration was quantified as follows: the percentage of severe eye degeneration phenotype in eyes expressing *SCA3^{polyQ78}* was set as 100% and compared to the percentage of severe eye degeneration upon downregulation of individual genes in astrocytes. An increased percentage of severe eye degeneration upon downregulation of a gene was indicated in orange (suppressor genes), and a decreased percentage of severe eye degeneration by downregulation of a gene was indicated in purple (enhancer genes). The darker the shade of the color of the enhancer or the suppressor, the larger the effect. The genes belonging to the Toll-Dif/Dorsal pathway are indicated in grey. Genes belonging to the IMD-Relish pathway are indicated in light green. Suppressor genes are marked in orange and enhancer genes are marked in purple. At least 80 eyes were counted for each condition. UAS-RNAi lines were randomly numbered and tested blindly by two people. Data present one time experiment.

confirmed that inhibition of these NF-κB pathway-associated genes in astrocytes delays neurodegeneration. These results underscore the relevance of astrocytes in the progression of SCA3^{polyQ78}-induced eye degeneration. Astrocytes in *Drosophila* share structural and functional similarities with mammalian astrocytes: amongst others, they provide trophic support to neurons and are involved in neurotransmitter recycling (reviewed in[51]). Thus, genes that we identified in our screen may be relevant for mammalian astrocytes as well.

Putative ways in which signaling from astrocytes can contribute to SCA3^{polyQ78}-induced eye degeneration

The candidate genes that we screened to study the astrocytes in SCA3 include (1) receptors that receive signals from neurons, (2) molecules that are involved in intercellular signaling and (3) molecules that can be secreted from the astrocytes. In this section, we will speculate how some genes can influence the extent of SCA3^{polyQ78}-induced degeneration.

Integrins

Our screen identified genes in astrocytes (*mew* and *if*) that encode conserved integrin subunits and can modulate SCA3^{polyQ78}-induced eye degeneration. *mew* was identified as an enhancer of SCA3, because RNAi of *mew* reduced the degenerative eye phenotype. Downregulation of another integrin gene (*if*) in astrocytes showed similar effects (Table 1). Integrins have widespread roles, such as cell growth, migration and inflammation. They predominantly interact with components of the extracellular matrix (ECM), but also with some cell surface proteins and microorganisms[52]. In our SCA3 model, integrins may also play a role in the targeting of astrocytes to the eye (Figure 4a). In eyes expressing SCA3^{polyQ78} but not in control eyes astrocytes are present (Figure 4). Whether integrins are involved in targeting astrocytes to SCA3^{polyQ78}-expressing eyes still needs additional investigation.

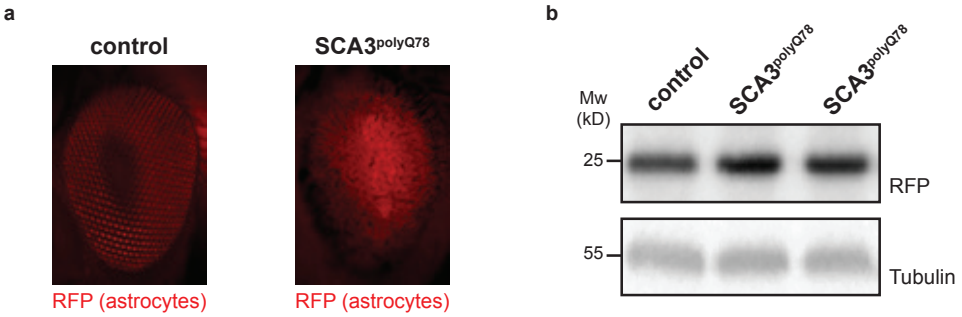

Figure 4. Eye-specific expression of SCA3^{polyQ78} results in the presence of astrocytes in the eye.
(a) In flies expressing SCA3^{polyQ78} in the eye, astrocytes were present in the eye, shown by the presence of astrocyte-specific expressed RFP. This astrocytes-specific RFP was not observed in the eyes of control flies in which the SCA3^{polyQ78} was not expressed . Figures are representative of at least three independent experiments. (b) Western blot showed that control and SCA3^{polyQ78}-expressing flies were expressing equal levels of RFP (right). The figure of western blot represents two experiments.
Genotypes in (a) and (b): control, *GMR-QF2/+; alrm-GAL4::UAS-myr-RFP/+*. SCA3^{polyQ78}: *GMR-QF2/+; alrm-GAL4::UAS-myr-RFP/ QUAS-SCA3^{polyQ78}*.

Integrins appear as heterodimers, and consist of two type I transmembrane proteins, an α subunit and a β subunit. In *Drosophila*, five α integrin subunits and two β integrin subunits have been identified. Our screen identified a conserved *Drosophila* integrin alpha subunit*, multiple edematous wings (mew)*, also known as *alpha PS1*, as an enhancer of SCA3 (Table 1): downregulation of *mew* in astrocytes reduced the SCA3^{polyQ78}-induced eye degeneration. Vertebrate orthologs of *mew* are the subunits α3, α6 and α7[53]. In addition, downregulation of another conserved alpha subunit, *inflated (if)*, also reduced the SCA3 ^{polyQ78}-induced eye degeneration, but to a lesser extent. *If* is orthologous to vertebrate α5, α8, αv and αIIβ[53]. In contrast, integrin α subunits *ItgaPS4* and *ItgaPS5* were identified as SCA3 suppressors, however, they have no ortholog in mammals. These data indicate cell non-autonomous contributions of α integrin subunits in astrocytes in SCA3, why some integrins are suppressors of SCA3 and other are enhancers is currently not clear.

There is some evidence that integrin signaling in astrocytes may play a role in neurodegeneration. In cultured primary rat astrocytes, stimulation with pro-inflammatory cytokine TNF-α increases rat astrocytic αv integrin expression[54]. Increased expression of αv integrin in astrocytes was also observed in a rat model of experimental autoimmune encephalomyelitis (EAE)[54]. Inhibiting αv integrin activity can alleviate symptoms of neurodegeneration: rat hippocampal slices incubated with beta-amyloid peptides display synaptic dysfunction, which was alleviated by addition of either a specific antagonist or antibody targeting αv integrin[55]. However,

a specific role of αv integrin in astrocytes in this study remains to be determined. Given that the αv integrin subunit can play a role in migration, phagocytosis and secretion of cytokines, it warrants further investigation in astrocytes.

Our results are in line with an enhancing role for αv integrin in neurodegeneration, as downregulation of αv integrin *Drosophila* ortholog *if* in astrocytes reduced the SCA3 phenotype. Together with the demonstration that another conserved *Drosophila* integrin alpha subunit (*mew*) was identified as an enhancer of SCA3, both *Drosophila* integrin alpha subunits, *mew* and *if* could be good candidates to investigate further to understand contributions of integrins in astrocytes to neurodegeneration.

GABA signaling

Another group of receptors that would be of interest to investigate further is the GABA receptors that respond to GABA, the main inhibitory compound in the CNS. Upon activation of the ionotropic GABA-A receptor with γ-aminobutyric acid (GABA), the receptor conducts chloride ions through its pore, thereby hyperpolarizing the neurons, thus having an inhibitory effect on neurotransmission in neurons (reviewed in[56]).

We identified *ligand-gated chloride channel homolog 3 (Lcch3)*, an ortholog of the mammalian inotropic GABA-A receptor subunit, as a suppressor of SCA3 (Table 1). *Drosophila* expresses three orthologs of the mammalian ionotropic GABA-A receptor subunit: *Lcch3*, *Resistant to dieldrin (Rdl)* and *Glycine receptor (Grd)*. Downregulation of each of them in astrocytes showed the enhanced severity of the $SCA3^{polyQ78}$-induced phenotype to a varying extent (Table 1). The similar effects of three GABA-A receptor genes on SCA3 may indicate that the GABA-A receptor in astrocytes plays a role in SCA3.

While studies examining astrocytic GABA-A receptor function in neurodegeneration are limited, prior research suggested that the astrocytic GABA-A receptor is involved in signaling between astrocytes and neurons. Addition of GABA to isolated hippocampal rat astrocytes or addition of a GABA-A receptor agonist resulted in an inward chloride current[57]. Furthermore, GABA resulted in an increase of intracellular calcium, possibly via voltage-gated channels. While the role of GABA signaling in astrocytes needs to be investigated further, it could indicate a means by which astrocytes can respond to inhibitory GABA signaling in degenerating neurons. Expression of the GABA-A receptor can be induced by exposure to proinflammatory cytokine IL-6, which can be secreted by glial cells after brain inflammation or injury. In a culture of rat astrocytes, GABA-A receptor expression is upregulated after stimulation with IL-6[58]. While these studies point at involvement of astrocytic GABA-A receptor activation and function during ND, its exact role remains to be demonstrated.

Receptors for DAMPs or cytokines on astrocytes

We did not find conclusive effects of astrocyte-specific downregulation of receptors, which either recognize DAMPs or cytokines.

Calcineurin

An important target of calcium-induced signaling is the serine/threonine phosphatase calcineurin. We examined calcineurin in our SCA3 model, given that calcium signaling plays an important role in glia and aberrant signaling has been associated with neurodegeneration[24]. Previous work showed that astrocytic calcineurin activity is associated with changes in morphology as well as neuroinflammation (reviewed in[24]). Calcineurin consists of a catalytic subunit A and a regulatory subunit B, both of which are required to respond to calcium.

There are three genes in *Drosophila* encoding the A subunit (*CanA1*, *CanA-14F* and *Pp2B-14D*). *CanA-14F* and *Pp2B-14D* are homologous to each other and probably arose as a result of gene duplication. There are two genes encoding the B subunit (*CanB* and *CanB2*). *CanA-14F* was identified as an enhancer of SCA3 (Table 1), because downregulation of *CanA-14F* in astrocytes reduced the SCA3^{polyQ78}-induced eye degeneration. Other calcineurin genes were not tested in our screen. Therefore, we tested whether the other isoforms of the calcineurin catalytic subunit could similarly contribute to SCA3 (Figure 5). Knockdown of *CanA1* in astrocytes also reduced the SCA3^{polyQ78}-induced eye degeneration. Similar results were found with downregulation of *Pp2B-14D* or *CanA-14F*, but the effects were less strong. Moreover, downregulation of both *Pp2B-14D* and *CanA-14F* attenuated the SCA3 phenotype. However, a gain of function or overexpression construct of a gene should have the opposite effect to the reduction of function by RNAi and provides additional evidence for involvement of a gene in SCA3. Indeed, expression of the active *Pp2B-14D* construct in astrocytes enhanced the SCA3^{polyQ78}-induced eye phenotype (Figure 5). Together, these data support a role for calcineurin in astrocytes as an enhancer of SCA3.

Our earlier studies provide evidence for a putative downstream target by which calcineurin can enhance activation of astrocytes: calcineurin activity can contribute to activation of NF-κB transcription factors (chapter 4)[59]. There is specificity of calcineurin with specific isoforms activating specific NF-κB transcription factor: the activity of CanA1 contributes to activation of NF-κB transcription factor Relish[59], activation of homologous Pp2B-14D/CanA14F can result in activation of NF-κB transcription factor Dif/Dorsal (chapter 4). However, we cannot exclude other calcineurin targets that account for their effects on SCA3. The effect of calcineurin on NF-κB is of particular interest, given that we also identified NF-κB transcription

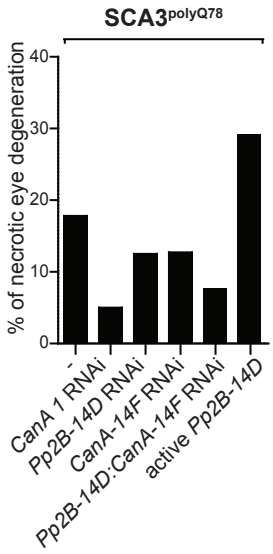

Figure 5. The effect of calcineurin in astrocytes on SCA3^{polyQ78}-induced eye degeneration. The fraction of necrotic eyes in SCA3^{polyQ78}-expressing eyes was compared to SCA3^{polyQ78} eyes expressing RNAi or overexpression constructs in astrocytes that target specific calcineurin isoforms. For each condition, at least 40 flies were examined. n=1. Genotypes: - (the SCA3^{polyQ78} control), *GMR-QF2/+; QUAS-SCA3^{polyQ78}::alrm-GAL4/+. CanA1* RNAi, *GMR-QF2/+; QUAS-SCA3^{polyQ78}::alrm-GAL4/+; UAS-CanA1 RNAi/+. Pp2B-14D* RNAi, *GMR-QF2/+; QUAS-SCA3^{polyQ78}::alrm-GAL4/UAS-Pp2B-14D RNAi. CanA-14F RNAi*, *GMR-QF2/+; QUAS-SCA3^{polyQ78}::alrm-GAL4/UAS-*CanA-14F RNAi. *Pp2B-14B:CanA-14F* RNAi, *GMR-QF2/+;QUAS-SCA3^{polyQ78}::alrm-GAL4/UAS-CanA-14F RNAi::UAS-Pp2B-14D RNAi.* Active *Pp2B-14D*, *GMR-QF2/+; QUAS-SCA3^{polyQ78}::alrm-GAL4/UAS-ΔPp2B-14D*[59].

factor Relish as an enhancer of SCA3.

Dysregulation of calcium signaling in astrocytes occurs in a mouse model of Huntington's disease, with astrocytes displaying spontaneous calcium signals, which may be associated with calcineurin activation[60]. Disturbances in calcium signaling and the resulting calcineurin activation by cleavage can occur in the brains of AD patients[61]. The cleavage of calcineurin occurs through the activation of calpain, a calcium-dependent protease[62]. We have preliminary evidence that suggests that calcineurin is activated in our SCA3 model: expression of SCA3^{polyQ78} in the eye resulted in the cleavage of calcineurin in lysates of fly heads (data not shown).

Other studies showed that calcineurin-dependent signaling in astrocytes contributes to their activation. Overexpression of calcineurin in astrocytes resulted in their activation and increased the expression of pro-inflammatory genes[63]. Our data are in line with a study, which showed that application of calcineurin inhibitor targeting astrocytes in the hippocampus attenuated synaptic dysfunction in AD mouse

model[64]. However, it is still a matter of debate whether the activation of calcineurin in astrocytes play a detrimental or beneficial role in NDs (reviewed in[24]).

Our current understanding of the interactions between astrocytes and neurons, which could possibly influence neuronal health are summarized in a model (Figure 6). Neuronal expression of an aggregation-prone protein results in a number of responses, including the release of neurotransmitters, pro-inflammatory cytokines and DAMPs. Aggregates can serve as DAMPs as well. Receptors for DAMPs include PGRPs (Peptidoglycan Recognition Receptors), and TLRs (Toll-like Receptors); however, integrins have been identified as receptors for DAMPs as well[65].

In our screen, we identified putative cell non-autonomous actions of astrocytes, which may contribute to neurodegeneration. Our setup of the screen is complementary to prior research where a misfolded, neurodegeneration-associated protein was expressed specifically in either neurons or astrocytes or ubiquitously in the brain (including neurons and astrocytes). Specific expression of aggregation-prone proteins in astrocytes also had detrimental effects on neurons[29,67]. Expression of misfolded, aggregation-prone proteins in astrocytes was also found in patients suffering from neurodegenerative diseases[68,69]. This expression may be detrimental to astrocytes (cell-autonomous) or alternatively, the resulting change in signaling in astrocytes may be detrimental to neurons (cell non-autonomous signaling). Our data are complementary to this research: we identify putative signaling events in astrocytes induced upon expression of misfolded proteins in neurons. Signals derived from cells (neurons or photoreceptors) are received by astrocytes. Subsequent signaling induced in astrocytes then results in signals that signal back to neurons (e.g. neuropeptides) or alternatively, affect levels of neurotransmitters (e.g. glutamate) that both influence neuronal functioning. In *Drosophila*, neuronal expression of $SCA3^{polyQ78}$ resulted in alteration of expression of genes in the head[70], suggesting the involvement of these genes in the responses to $SCA3^{polyQ78}$. However, how gene expression in astrocytes is altered still needs further investigation. Previous studies have identified genes of interest that are expressed in SCA3 *Drosophila* models, including NF-κB, ligands for receptors acting upstream of NF-κB, as well as transcriptional targets of NF-κB[70,71]. We also observed downregulation of EAAT1, a glutamate transporter (prelim. data), suggesting alterations in glutamate levels.

The effect of genes that were identified in our SCA3 eye model can be tested in flies expressing $SCA3^{polyQ78}$ in neurons to verify whether these genes in astrocytes also have similar effects when $SCA3^{polyQ78}$ is expressed in neurons. In chapter 3, we indeed verify that a subset of genes in astrocytes we tested in our neuronal model also modulated the effects of neuronal SCA3. This underscores the relevance of our SCA3 eye model for neurodegenerative diseases.

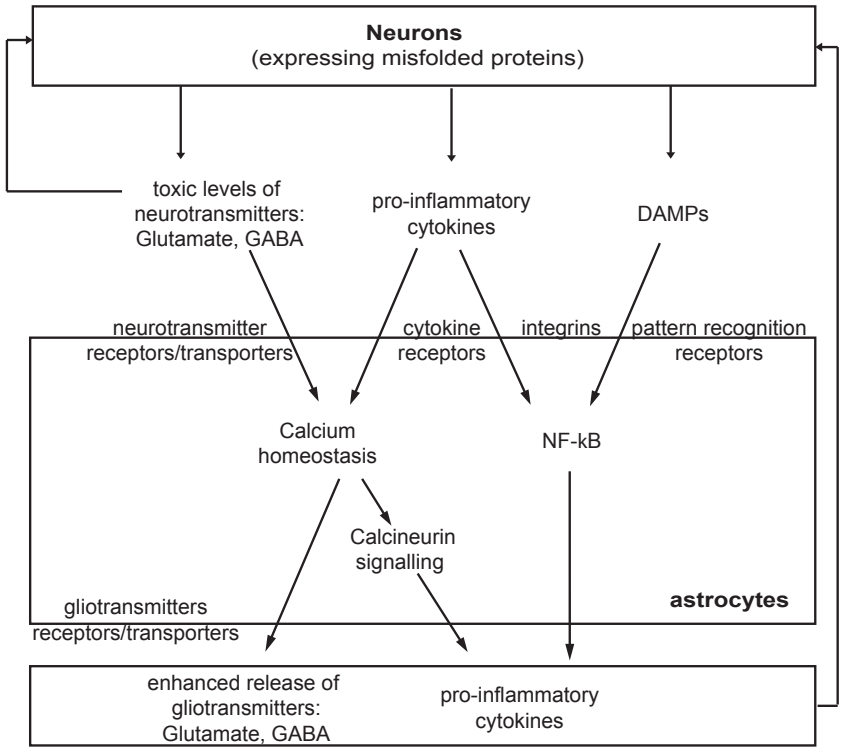

Figure 6. Model for molecules and signaling pathways that may be involved in neuron-astrocyte interactions in NDs. Neurons that express misfolded proteins as occurs in NDs can release DAMPs, pro-inflammatory cytokines or elevated levels of neurotransmitters. These molecules are recognized by different receptors on astrocytes, resulting in the activation of intracellular signaling pathways. This includes elevation of intracellular calcium and activation of calcineurin, as well as activation of NF-κB signaling, which can occur downstream of calcineurin[59,66]. The activation of intercellular signaling results in the generation of molecules that are released from astrocytes, such as pro-inflammatory cytokines and gliotransmitters which may have negative effects on neurons.

MATERIALS AND METHODS

Drosophila strains

All flies were raised at 25 degrees and cultured on standard fly food. *GMR-QF2* (stock number 59283), *alrm-GAL4* (stock number 67031) were obtained from Bloomington Drosophila stock center (BDSC, Bloomington, Indiana, U.S.A.). Information on additional fly lines can be found in the materials and methods section in chapter 3. For the screen, all the RNAi fly stocks we used were mentioned in the screen table (Table 1) and were obtained from Vienna Drosophila Research Center (VDRC) or Bloomington. RNAi lines targeting calcineurin genes as well as a fly line expressing active calcineurin have been described previously[59,66]. Generation of *QUAS-SCA3^{polyQ27}* and *QUAS-SCA3^{polyQ78}* are described in chapter 3). All the transgenic fly lines were used were made in the w1118 background.

Genetics

To independently and simultaneously manipulate gene expression in eyes or astrocytes, we used the QF-QUAS system to express constructs in neurons or in eyes and UAS-GAL4 to manipulate gene expression in astrocytes (using *alrm*-GAL4).

To screen for involvement of astrocyte-associated genes in the SCA3^{polyQ78}-induced eye degeneration we used the following fly line: *GMR-QF2*/(Y); *QUAS-SCA3^{polyQ78}:: alrm-GAL4*/*CyO-tub-QS*. In this line, expression of SCA3^{polyQ78} is suppressed by QF2 suppressor QS[72]. To analyze the effect of gene expression in astrocytes on the SCA3 eye phenotype, we crossed this line to different UAS constructs. To quantify eye degeneration by analyzing levels of mCD8-GFP, we used the line *GMR-QF2*/(Y); *QUAS-SCA3^{polyQ78}* /*CyO-tub-QS: alrm-GAL4: QUAS-mCD8-GFP*, and crossed them to UAS lines or w1118 flies. The fly line *gmr-QF2*/(Y); *QUAS-SCA3^{polyQ78}:: alrm-GAL4*/*CyO-tub-QS* was crossed to individual *UAS-RNAi* lines to obtain astrocyte-specific knockdown of the indicated genes (genotype of the cross *GMR-QF2/+; QUAS-SCA3^{polyQ78}: alrm-GAL4/+* together with *UAS-RNAi/+*). To prevent bias, UAS-RNAi lines were randomly numbered and tested blindly.

Analysis of eye degeneration

At least 40 2-day old flies were collected for each fly line that was examined. The degenerative eye phenotypes of the flies were examined under a dissecting microscope and two persons scored them independently. To prevent bias, UAS-RNAi lines were randomly numbered and tested blindly. The numbers of the mild

and severe SCA3 eye phenotype were recorded and the fraction of each eye phenotype was calculated.

Immunofluorescence staining

Fly heads were fixed with 3.7% formaldehyde (Sigma Aldrich) for 15 min, washed 3 times in PBS. Then brains were dissected in PBS, and fixed again in 3.7% formaldehyde for 10 min, followed by washing 5 times with PBS-T (0.1% Triton-X-100). Fly brains were next blocked with 2% BSA /0.1% Triton-X-100 in PBS for 1 h. After washing 3 times with PBS-T, brains were incubated with DAPI (1:100 in 2% BSA PBS-T) for 40 min and washed with PBS-T 3 times. Brains were mounted on the microscope slide. Fluorescent images were obtained with a confocal microscope, SP8 (Leica DMI 6000).

Western Blotting

For each condition, at least 30 two-day-old flies were collected and frozen in liquid nitrogen. Flies were decapitated by vortexing. Fly heads were lysed in the laemili buffer by sonification. An equal amount of samples were loaded on 12.5% SDS-PAGE gels. The antibody was used were rat anti-HA-Peroxidase (1:1000, Roche Diagnostics GmbH, Germany), mouse anti-RFP (1:1000, Chromotek 6G6) and mouse anti-alpha tubulin (1:2000, Sigma T5138). The membranes were detected in Chemi Doc™ Touch (Bio-Rad). The intensity of the bands was analyzed by using Image J.

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