Glia in Alzheimer's disease and aging: Molecular mechanisms underlying astrocyte and microglia reactivity

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CHAPTER 3

PROTEASOME ACTIVITY REGULATES GFAP EXPRESSION IN ASTROCYTES VIA THE NOTCH PATHWAY

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*In preparation*

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Astrocytes are crucial cells for proper neuronal functioning in the developing and adult brain. Glial fibrillary acidic protein (GFAP) is the main and characteristic intermediate filament (IF) protein in astrocytes. Expression of GFAP is highly regulated in reactive astrocytes, upon brain damage and in neurodegenerative diseases. The molecular mechanisms for GFAP upregulation, and the role of the enhanced IF protein levels in the astrocyte’s response to brain insults are still not completely elucidated. In this context, Notch signaling emerges as an important regulator of GFAP expression in reactive astrocytes. Here, we demonstrate that an increase in proteasome activity is a potent stimulus for GFAP expression. The proteasome is one of the major protein degradation systems in the cell and its dysregulation is implicated in many brain diseases. We screened several molecular compounds for their ability to increase proteasome activity in astrocytes, in vitro, and found two compounds—an opioid receptor antagonist and a purinergic receptor antagonist. The increase in proteasome activity was paralleled by an increase of immunoproteasome subunit expression and, importantly, an increase in GFAP expression. By using specific inhibitors for the immunoproteasome and Notch activity showed that both of this compounds, independently, could prevent the proteasome mediated GFAP activation. These results further strengthen our earlier reported link between the proteasome and GFAP regulation and indicate that the immunoproteasome activity and Notch signaling are vital players in this regulation. This study brings us one step closer to understand the molecular mechanisms underlying astrocytic GFAP regulation in the healthy and injured brain.
INTRODUCTION

Astrocytes represent the major population of glial cells in the adult mammalian brain. After brain damage and in neurodegenerative diseases, astrocytes respond to the injury by a process termed ‘reactive gliosis’. This involves morphological and molecular changes, including an upregulation of IF proteins among which the astrocyte specific GFAP IF protein [35, 47]. The molecular pathways underlying the upregulation of GFAP and the downstream effects of this upregulation are not fully understood. Previously, our group showed that proteasome activity is involved in the regulation of GFAP transcript levels in astrocytes [157]. The ubiquitin proteasome system is the main protein degradation system in the cell. It plays a key role in degrading aberrant and damaged proteins. In addition, it is involved in a variety of other vital cellular processes, such as regulation of the cell cycle, cellular differentiation, and signal transduction; likely via degrading short-lived proteins such as cell cycle proteins and transcription factors [79, 189]. The proteolytic complex of the proteasome - the 20S core - is a barrel shaped dimeric complex composed of two rings of seven, non-identical, proteolytic β-subunits together with two rings of seven, non-proteolytic α-subunits [86]. The inner β-rings contain active sites attributed to β1 (caspase-like), β2 (trypsin-like), or β5 (chymotrypsin-like) catalytic subunits [87, 88]. The immunoproteasome is a variant of the constitutive proteasome and is mainly induced by inflammatory signaling. It harbors slightly different catalytic β subunits (LMP2, β 1i, Psmb8), MECL1 (β2i, Psmb10), (LMP7, β5i, Psmb9) compared to its constitutive counterparts, making the immunoproteasome more suitable for MHC class I antigen generation [94, 96]. In addition to peptide generation for MHC, the immunoproteasome has also been suggested to be directly involved in regulation of inflammatory signaling [80]. Neuroinflammation is a characteristic of neurodegenerative diseases, such as AD (reviewed in [190]). In human AD brain tissue we have observed an increased expression of immunoproteasome subunits and their proteolytic activity. The β1i and β2i subunit immunoreactivity was more intense in reactive astrocytes marked by elevated levels of GFAP [7][chapter 2]. In view of its role in gene expression and in inflammation we hypothesized that an enhanced immunoproteasome activity underlies the increase in GFAP expression in reactive astrocytes. Previously
we have shown that the inhibition of total proteasome activity in astrocytes leads to a strong downregulation of GFAP mRNA expression [157].

Another important regulator of GFAP expression is Notch signaling (reviewed in [191]). Notch is a key regulator of cell fate decisions of neural stem cells during development, and Notch signaling induces astrocyte differentiation via stimulation of GFAP expression [192]. The expression of Notch persists in the adult brain in mature astrocytes [107, 193][chapter 4]. In reactive astrocytes, key developmental pathways, such as Notch signaling, are activated (reviewed in [194]). This Notch activation might be involved in the upregulation of GFAP (reviewed in [193]). Interestingly, Notch signaling was shown to be activated by proteasomal activity through degradation of its negative regulator Numb [195].

In this study we investigated the effect of proteasome activation on reactivity of astrocytes, as measured by an increased GFAP expression and an increased immune signaling. We screened a number of small molecules for their ability to activate the proteasome in astrocytes, and subsequently assessed their role in the activation of the immunoproteasome and the regulation of GFAP expression. Moreover, we studied whether Notch signaling is involved in the process of GFAP regulation upon proteasome activation.

**MATERIALS AND METHODS**

**Cell culture**

All cells were cultured at 37°C under a humidified 5% CO₂ / 95% air atmosphere. DBT, mouse astrocytoma cells, have a stable GFAP expression [196] and were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and an antibiotic mixture of 1% penicillin/streptomycin (all Invitrogen, Carlsbad, CA).

**Cell treatments**

For treatment with the proteasome activator compounds, the cells were plated at a density of 10,000 cells per well (24-well plate), 8 hour prior to treatment. Subsequently,
the cells were treated with the proteasome activators #1-9 at 5μM for 24h or the corresponding concentration of mock control (dimethyl-sulfoxide (DMSO)), followed by assessment of proteasome activity, protein expression (immunocytochemistry), or mRNA expression (quantitative real-time PCR). For total proteasome inhibition, cells were treated with MG132 40 μM overnight (Affinity Research). Specific inhibition of the β5i subunit was performed with 200nM of the β5i inhibitor ONX0914 (previously named PR-957). For inhibition of Notch activity, DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl (Sigma-Aldrich Corp.) was dissolved in DMSO and added to the cells with a final concentration of 250nM for 24 hours. DMSO (final concentration of 0.3%) was applied in the control condition.

**Proteasome activity, immunocytochemistry and image analysis.**

After treatment of cells with the different proteasome activators, the cells were washed with phosphate buffered saline pH 7.4 (PBS) followed by incubation with BODIPY-epoxomicin, a cell permeable proteasome activity probe [197] at 300nM at 37°C in a 5% CO$_2$ incubator for 1h. After probe incubation the cells were washed with PBS, pH 7.4 and fixed in 4% paraformaldehyde in PBS, pH 7.0, at room temperature for 10 minutes. Cells were blocked in PBS, supplemented with 10% normal donkey serum and 0.4% Triton X-100, incubated overnight with anti GFAP (rabbit polyclonal, DakoCytomation, Z0334, final concentration: 1:2000) antibody, diluted in 3% normal donkey serum and 0.4% Triton X-100 in PBS at 4°C. For fluorescence visualization, the cells were incubated in with Alexa 488 conjugated secondary antibody (Jackson Immunoresearch Laboratory Inc, final dilution: 1:700), and Hoechst as a nuclear dye in PBS at room temperature for 1h. After washing 500 μl PBS was added to the wells and images were obtained using a Zeiss Axiovert 135M inverted microscope and a Sony XCD-X700 digital camera system. Two images at 10x magnification per treatment condition were taken. The same exposure time was used for DMSO controls and activator treated cells for both the proteasome activity probe (Cy3 channel) and the GFAP (Alexa 488 channel). The Image J software was used to extract and measure the gray values obtained from both channels. The mean gray value intensity was used for quantification of probe intensity (proteasome activity).
and GFAP expression. Adobe Photoshop was used to arrange the TIFF files for presentation and Image J was used for calculating and measuring gray value intensity.

**RNA isolation, cDNA synthesis and quantitative real-time PCR**
For RNA isolation, cells were harvested and total RNA was isolated with Trizol (Invitrogen) according to the manufacturer’s protocol. The resulting RNA pellet was dissolved in RNAse free water. The RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). The reverse transcriptase and real-time quantitative PCR reactions as well as the quantification and normalization procedures were performed as previously described [6]. Primers used in this study are listed in see Supplemental table 1.

**Ingenuity pathway analysis of GFAP regulation pathways**
To find genes/ proteins involved in GFAP regulation together with regulation of Notch (Hes1), Tgfβ, Immunoproteasome (Psmb8) and activator # 2 pathways we performed an Ingenuity pathway analysis. Molecules involved in regulation of each of these different pathways were obtained from Ingenuity Pathway Analysis (IPA, Ingenuity systems, Qiagen, USA). Subsequently, these lists were overlaid and molecules that were involved in GFAP and in regulation of at least two of the other above-mentioned pathways were shown in (Fig. 7C).

**Statistical analysis**
Data was analyzed using GraphPad Prism software (GraphPad Software). Significance was assessed using a non-parametric Mann–Whitney test. Differences existed amongst groups were considered significant at p < 0.05.

**RESULTS**
**Increased proteasome activity correlate with increased GFAP expression**
A small library screen was previously performed (Ovaa, unpublished data) to identify
molecules that have an ability to increase the proteasome activity in various cell types. In the current study, we tested 12 potent compounds from the initial screen for their ability to activate the proteasome in an immortalized mouse astrocytoma line. The astrocytes were treated with each compound at a concentration of 5 μM for 24h; these conditions were based on previous studies (Ovaa, unpublished data). Subsequently, the proteasome activity was assessed in living cells by adding a cell permeable, fluorescently labeled (Bodipy (-Cy3)) proteasome activity probe to the cells, as described before [7, 197][chapter 2]. The cells were then immunolabeled for GFAP followed by quantification of both the probe and the GFAP labeling intensity. Out of the 9 compounds #2 and #4 (black fill), an opioid receptor antagonist and a purinergic receptor antagonist, respectively, showed an ability to enhance the proteasome activity in the astrocytes (Fig. 1A, D-F). Both of these (black fill) compounds also showed the strongest GFAP labeling compared to the other compounds (Fig. 1B, G-I). Analysis of the proteasome activity and GFAP staining intensity for all the 9 compounds revealed a positive correlation between proteasome activity and GFAP protein expression (Fig. 1C). GFAP expression and proteasome activity seems to increase in a linear way with small increases in proteasome activity; larger increases in proteasome activity appear not to increase GFAP expression further. All 9 molecules were also screened for their effect on GFAP mRNA expression levels. In accordance with the proteasome activity and GFAP staining, both activators #2 and #4 were able to increase Gfap mRNA transcript levels, while the other activator compounds had only little effect on Gfap mRNA expression (Fig. 2A). After this initial screening process, we proceeded with activator #2 and #4 to further assess their capacity to regulate different GFAP isoforms; we showed in an earlier study that the canonical isoform Gfapα and an alternative splice variant of GFAP, Gfapδ, are both upregulated in reactive astrocytes [198]. The expression of the GFAP isoforms, was determined 24h after treatment with activator #2 and #4. Both activators had a clear effect on Gfapα expression and caused about a three-fold increase compared to controls (Fig. 2B), while no effect was observed on the expression of Gfapδ (Fig. 2C).

In summary, we identified two compounds, activator #2 and #4 that both showed an ability to increase proteasome activity in astrocytoma cells in vitro. This
increase in proteasome activity also led to a significant increase in GFAPα mRNA expression and a more intense GFAP staining.
Increased expression of the immunoproteasome in response to proteasome activation

In order to explore whether induction of the constitutive or immunoproteasome could be responsible for the increase of proteasome activity, we analyzed the gene expression of the different proteolytic subunits of the proteasome in response to activator #2 or #4. None of the two activators had an effect on the expression of the proteolytic subunits of the constitutive proteasome (β1, β2, β5; Fig. 3A). However, treatment with activator #2 led to an enhanced expression of all immunoproteasome subunits (β1i, β2i, β5i) compared to the control with a 3.4-, 1.6-, and 10.3-fold increase, respectively; while activator #4 lead to a 4.2-fold increase in β1i, and an 11.2-fold increase in β5i expression (Fig. 3B). The endogenous expression of these subunits in untreated cells is very low, and is only induced upon activator treatment. Previously, our group showed that gene expression of immunoproteasome subunits corresponds well with their activity [7][chapter 2]. Hence, an increase of the immunoproteasome transcript levels may underlie the increase in proteasome activity upon treatment with activator #2 and #4.

Figure 2. Activation of the proteasome enhances Gfap expression.

(A) The activators were screened for their ability to modulate Gfap mRNA expression in cultured astrocytes. The cells were treated with the activators at 5μM for 24h before isolation of mRNA and assessment of GFAP expression (n=3). (B) Follow-up experiments were done to assess the mRNA expression of two different GFAP isoforms (B) Gfapα and (C) Gfapδ, separately, upon treatment with activator #2 and #4. Both substances increased the expression of Gfapα, while Gfapδ expression remained stable (n=4). Bars depict mean and error bars depict SEM, * p < 0.05.

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Astrocytes increase anti-inflammatory signaling in an immunoproteasome dependent manner

The immunoproteasome is suggested to play an important role not only in generation of peptides for MHC class I presentation, but also in innate immune signaling [7, 80, 199][chapter 2]. Previously, inhibition of the immunoproteasome activity in microglia and blood monocytes has been shown to significantly reduce the induction of proinflammatory innate immune molecules [7, 199][chapter 2]. Therefore, we here asked whether the activator #2- or #4-induced increase in immunoproteasome expression, would lead to an increased expression of pro-inflammatory molecules.

Figure 3. Proteasome activators increase the expression of immunoproteasome subunits. The mRNA expression of proteasome subunits was analyzed after activator #2 and #4 treatment of the astrocytes. (A) Graphs show the expression of the constitutive proteolytic proteasome subunits, β1, β2 and β5 and graphs in (B) show the expression of the proteolytic immunoproteasome subunits β1i, β2i and β5i. Bars depict mean and error bars depict SEM, * p < 0.05, n=4-5.
Interestingly, the expression of the pro-inflammatory molecules, Il6 and Tnfα did not significantly change upon activator stimulation; Il6 rather showed a trend for a decreased expression (Fig. 4 A-B). On the contrary, expression of the anti-inflammatory molecules Tgfb and Il4 were increased after treatment with activator #2 and #4 (Fig. 4 C-D). Il4 expression showed a clear albeit non-significant trend for an upregulation upon activator #2 treatment.

**Induction of Gfap expression upon proteasome activation is prevented by inhibition of β5i immunoproteasome activity**

Next, we assessed whether activation of the β5i immunoproteasome is of crucial importance for an upregulation of Gfap expression upon proteasome activation, using activator #2. It has previously been shown in our lab that a total inhibition of proteasome activity leads to a rapid and near complete reduction of Gfap transcript levels in human astrocytoma cells [157]. To address the question whether an increase in β5i activity is vital for GFAP upregulation upon activator #2 treatment, we inhibited the β5i immunoproteasome activity using ONX0914 - a molecule specifically targeting the β5i immunoproteasome activity [199], in samples treated with activator #2. The astrocytes were incubated with activator #2 overnight, to activate the immuno-
Figure 5. Inhibition of the immunoproteasome abolishes activator # 2 induced Gfapα and Tgfb expression. (A) Treatment scheme for inhibition of the immunoproteasome using ONX0914 in cells treated with activator #2; astrocytes were first treated with activator #2 alone (5μM) before the ONX0914 inhibitor (200 nM) was added for the last 6h of treatment. (B-C) Inhibition of the immunoproteasome using ONX0914 reduced activator #2 induced proteasome activity in astrocytes as shown by image analysis and flow cytometry. (B) Shows representative images of astrocytes treated with mock (left), activator #2 only (middle), or a combined treatment with activator #2 and ONX0912 (right), followed by a 2h incubation with the proteasome activity probe. The graph shows intensity quantification of the probe fluorescence, bars show probe intensity as fold over mock controls derived from 3 tiled images. (C) Shows the
proteasome, followed by addition of the irreversible β5i inhibitor for the last 6 hours at a concentration previously used in earlier studies [7, 199][chapter 2]; for treatment scheme see (Fig. 5A). We used both imaging of cells and flow cytometry as readout of the proteasome activity. Our data shows that inhibition with ONX0914 reduced the proteasome activity of the activator # 2 treated astrocytes, to a level comparable to that of mock treated controls (Fig. 5 B-C). The expression levels of Gfapα followed the pattern of the proteasome activity; cells treated with the β5i inhibitor together with activator #2 showed a reduced Gfapα expression compared to cells treated with activator #2 alone (Fig. 5D). The Gfapα expression in the inhibited, activator # 2 treated samples was more comparable to that of mock treated controls. The expression of Gfapδ was not affected by any of the treatments (Fig. 5E). Interestingly, inhibition of the β5i activity in the activator #2 treated samples did not prevent the upregulation of Tgfb (Fig. 5F). Inhibition of β5i alone without activating the proteasome did not affect Gfapα, Gfapδ, or Tgfb mRNA expression (Supplemental. Fig. 1). Moreover, we used the general proteasome inhibitor MG132 as a positive control to show that total block of the proteasome activity abolishes expression of Gfapα as shown previously [157] (Fig. 5D). Taken together, here we show that Gfapα is increased upon (immuno)proteasome activation and decreased upon proteasome inhibition, demonstrating a strong correlation between Gfapα expression and proteasome activity. In contrast, regulation of Gfapδ expression remained stable upon modulation of proteasome activity (Fig. 5E). Importantly, in astrocytes with enhanced proteasome activity induced by activator #2 treatment, specific inhibition of β5i prevented the increase of Gfapα expression, but did not compromise the increase of the expression of the anti-inflammatory signaling molecule Tgfb (see scheme Fig. 7A). Interestingly, MG132, on the contrary to ONX0914 caused a marked reduction of Tgfb expression (Fig. 5F).

flow cytometric data of the probe intensity in cells treated as described in (A), quantification of the geometric mean of the probe intensity is shown in the bar graph. (D-F) Gene expression of Gfapα (D), Gfapδ (E), and Tgfb (F) in astrocytes treated with vehicle (Control), the proteasome inhibitor MG132, activator #2, or activator #2 together with ONX0914 as described in (A). Bars show normalized expression as fold over mock treated control, error bars show SEM. * p < 0.05, ** p < 0.01 compared to controls, ### p< 0.001 compared to activator # 2, n= 5-7.
Proteasome activation promotes Notch signaling in astrocytes

Notch signaling is regulated by proteasome activity [195], and has been shown to be an important regulator of astrocyte reactivity [200]. Notch activation has been discussed before to be responsible for the upregulation of GFAP in reactive astrocytes (reviewed in [193]). Based on this hypothesis, we investigated whether proteasome activation promotes Notch signaling in astrocytes.

Figure 6. Inhibition of Notch activity prevents activator # 2 induced Gfap and Tgfβ expression. Gene expression of the Notch target Hes-1 (A), Gfapα (C), and Tgfβ (D) in cells simultaneously treated with the Notch inhibitor DAPT and activator # 2. (B) Shows Hes-1 expression after treatment of activator #2 together with the β5i-inhibitor ONX0914 for the last 6h of the 24h activator treatment. Bars show normalized expression, as fold over mock treated control, error bars show SEM. * p < 0.05, ** p < 0.01 compared to controls, # p < 0.05, ## p < 0.01 compared to activator # 2, n=5-7.
activation could induce Notch signaling, which in turn, could stimulate Gfap expression.

First, we analyzed the regulation of Notch signaling upon activation of the proteasome. To this end, we activated the proteasome with activator #2 and analyzed the expression of the Notch downstream target gene Hes-1, as readout for Notch activity. Stimulation of proteasome activity significantly enhanced expression of Hes-1, a sign for activation of the Notch pathway. The upregulation of Hes-1 was dependent on Notch activity since the inhibition of the pathway using the γ-secretase inhibitor DAPT - a potent inhibitor of the Notch pathway - reduced Hes-1 expression levels induced by activator #2 significantly, thus counteracting the upregulation of Hes-1 induced by activator #2 (Fig. 6A). Treatment of cells with DAPT and activator #2 was performed simultaneously. As argued above, the mechanism of activator #2 may work via an activation of the immunoproteasome, which would predict that β5i inhibition (ONX0914) could block the activator #2 dependent increase of Hes-1. As shown in (Fig. 6B), ONX0914 treatment was indeed sufficient to counteract the effect of activator #2 and reduced Hes-1 expression significantly (Fig. 6B), while ONX0914 treatment alone did not have any significant effect on Hes-1 expression (Supplemental. Fig 1). To conclude, these data show that Notch activity is indeed regulated by proteasome activity in astrocytes. Importantly, inhibition of the immunoproteasome is sufficient to prevent Notch activation (Fig. 7A-B).

Based on its regulatory role on GFAP expression, we here investigated whether Notch activity is necessary for the upregulation of Gfapα upon proteasome activation. The Gfap expression was determined in cells simultaneously treated with activator #2 and the γ-secretase inhibitor DAPT. Intriguingly, the astrocytes displayed reduced Gfapα expression compared to cells treated with activator #2 alone and comparable expression levels to control cells (Fig. 6C). Thus, when inhibiting the activator #2-mediated induction of Notch signaling, the upregulation of Gfapα expression was also prevented. These data show that activation of the Notch pathway is necessary for the observed Gfapα induction upon proteasome activation, indicating that the Notch pathway is an integral component of the regulatory cascade initiated by proteasome activation (see scheme Fig. 7A-B). In addition, inhibition of Notch activity in cells treated with activator #2 showed some preventive effect (al-
though not significant, \( p = 0.11 \) on the induction of Tgfb (Fig. 6C).

A pathway analysis was performed using the ingenuity pathway analysis software to find common interaction targets between immunoproteasome activation, GFAP, Notch signaling, Activator #2 (opioid receptor interactions/signaling), and Tgfb. This analysis revealed that the immune molecules TNFα and LPS (gray molecules) interact with all pathways, while the ubiquitin protein C (UbC), involved in protein degradation, and the transcription factor STAT3 are interacting with all pathways apart from TGFβ (green molecules). Three molecules, the hormone beta-estradiol, the transcription factor Ap-1 and the immune molecule IL-6 (blue molecules) were found to interact with GFAP, TGFβ and two other pathways (Fig. 7C).

**DISCUSSION**

Reactive gliosis is mainly characterized by increased GFAP expression within astrocytes in the brain. However, the precise molecular mechanism underlying this GFAP induction remains poorly understood. In this study we activated the proteasome with two small molecular compounds in astrocytes, which resulted in an increase in GFAPα expression. These result are in accordance with previous findings from our lab showing a reduced GFAP expression upon inhibition of the proteasome [157], and further strengthen the link between proteasome activity and GFAP regulation.

**Downstream effects of proteasome activation in astrocytes**

In contrast to proteasome inhibitors, little is known about molecules that have a stimulating effect on proteasome activity, and even less is known about the downstream effects in the cell upon proteasome activation [201]. In this study, we show that, in cultured astrocytes, agonists for purinergic or opioid receptors activate the proteasome and increase GFAP expression. In addition, the agonists increase expression of the anti-inflammatory molecule Tgfb. Interestingly, the GFAPα expression was reduced upon inhibition of the β5i immunoproteasome activity and Notch activity, while, on the contrary, the elevated Tgfb expression was maintained in the presence of specific β5i, or Notch inhibition (see scheme Fig. 7A-B). The pathway analysis performed in this study (Fig. 7C) revealed that GFAP, Notch, the immunoproteasome,
and the opioid receptor are all connected via STAT3, which suggests that this transcription factor may play an important role in proteasome mediated GFAP regulation. Activated astrocytes show a fast and long lasting STAT3 activation after spinal cord injury, leading to increased GFAP expression, cell size and an anti-inflammatory phenotype [202]. Moreover, inactivation of STAT3 decreases GFAP expression [203,

Figure 7. Schematic overview of the regulation of Gfapα and Tgfb upon proteasome activation via the Notch pathway in astrocytes.
(A-B) Display a schematic overview of the regulation of Gfapα and Tgfb upon proteasome activation that is dependent on (A) inhibition of the immunoproteasome, or (B) inhibition of Notch signaling. (A) Activation of the proteasome leads to an increase in Notch activity and, in the condition of the activation of the Notch pathway, an increase of Gfapα and Tgfb expression. As shown in literature and depicted in orange, Tgfb signaling potentiates the expression of GFAP [207]. Sustained high GFAP levels, in a negative feedback loop, decrease Notch signaling (Orre et al., submitted; Kanski et al. in prep.). When inhibiting the immunoproteasome with a β5i inhibitor, the activator #2 induced upregulation of Gfapα, but not Tgfb expression was abolished. (B) Shows a schematic overview of the regulation of Gfapα and Tgfb upon proteasome activation in the presence of the Notch pathway inhibitor DAPT.
204], thus these data link STAT3 to both early and sustained activation of astrocytes. In addition, inhibition of β5i activity leads to decreased STAT3 phosphorylation and activation in T-cells [205]. Phosphorylation activates STAT3, thus β5i activity might play a role in activation of STAT3 signaling. Finally, STAT3 activity is also increased by Notch signaling [206], emphasizing the regulatory role of Notch in GFAP expression. A total block of the proteasome activity did not significantly reduce STAT3 mRNA expression [157], but showed a clear trend. Here, we, moreover, show that Tgfβ expression is tightly following the GFAP expression after stimulation with the proteasome activators; also TGFβ has previously been linked to regulation of GFAP through the JAK/STAT pathway. TGFβ activates SMAD signaling interacting with STAT3 that, in turn, regulates GFAP expression [207], see scheme (Fig. 7A-B). The transcription factor AP-1 was also present among the molecules involved in GFAP regulation from the pathway analysis (Fig. 7C). AP-1 is a dimer made out of proteins from the Fos and Jun family [208]. Total inhibition of proteasome activity, by Epoxomicin led to an increase in c-jun expression in human astrocytes while GFAP expression was decreased [157]. A recent study, investigating the effect of transcription factor mutations for their capacity to alter the activity of the GFAP promoter in mouse, showed that mutations in STAT3 led to a strong reduction in GFAP promoter activity, while mutation in AP-1 had little effect on the GFAP promoter activity [209]. In addition, based on the pathway analysis, AP-1 was not linked to the immunoproteasome (β5i), which indicates that the immunoproteasome asserted influence on GFAP expression is more likely to be mediated by STAT3 activation than AP-1 activation.

Regulation of GFAP isoform expression upon proteasome activation

Activation of the proteasome activity upon activator #2 treatment increased GFAPα whereas expression levels of GFAPδ remained stable. These results are in line with previous findings; an increase in activity of the GFAP promoter, encoded in a GFAP minigene, predominantly led to an increase in the expression of GFAPα, while the induction of alternative GFAPδ transcripts is less pronounced [210]. Strong splice sites are present in the exons of GFAPα, while the splice sites for GFAPδ are weaker; therefore, when competing for the recruitment of the same splicing factors - during rapid transcription - there is not enough time for the recruitment of splicing factors
to the weak splice sites (discussed in Kanski et al., in preparation). This can explain why astrocytes that are acutely activated by, for example, the proteasome activators show an increase in GFAPα expression while the GFAPδ expression remains stable. Conversely, inhibition of the proteasome activity with a reversible inhibitor such as MG132 might slow down transcription of GFAP resulting, as shown in this study, in a downregulation of mainly GFAPα. In contrast, a total block of proteasome activity using the irreversible inhibitor Epoxomicin might completely block GFAP transcription and as a result all GFAP isoforms are silenced [157]. In reactive astrocytes in the diseased brain, a chronic and sustained activation of GFAP transcription may provide enough time allowing upregulation of all GFAP isoforms [198].

**Inhibition of Notch activity prevents induction of GFAP**

As demonstrated in this study, the regulation of Gfap expression upon proteasome activation is dependent on Notch activity. Notch signaling is an important regulator of GFAP expression in newborn astrocytes via epigenetic remodeling of the Gfap promoter [192]. Interestingly, it becomes more and more clear that Notch can also regulate the reactivity of differentiated astrocytes [200], where it might induce an upregulation of GFAP (reviewed in [193]). Here we show that blocking the increase in Notch activity prevents GFAP induction, demonstrating that proteasome activation, Notch signaling, and GFAP expression are tightly interconnected (see scheme Fig. 7A-B). Previously, it has been shown that inhibition of Notch in LPS-treated astrocytes prevented upregulation of GFAP, and decreased the expression of pro-inflammatory cytokines through blocking the NFκB and partially the JAK/STAT pathway [200]. Thus, the Notch signaling pathway represents an interesting therapeutic target to limit reactive gliosis. However, due to its pleiotropic functions in the brain, a general inhibition of Notch is likely to have severe side effects. Here we observed that inhibition of the immunoproteasome is sufficient to reduce the increased Notch activity in activator #2 treated astrocytes to that of control cells. How the immunoproteasome regulates Notch activity is still unknown. An indirect blockage of Notch activity via inhibition of the immunoproteasome might represent a tool to specifically inhibit upregulation of Notch in activated glia. Maintenance of Notch activity in neurons could significantly limit side effects in comparison to general Notch inhibition.
Modulation of the (immuno)proteasome, a tool to modulate astrocyte phenotypes?

Molecules that can activate the proteasome may be attractive as a treatment strategy for neurodegenerative diseases. As discussed earlier [82], accumulation of oxidatively damaged and misfolded proteins in neurons has been found in several neurodegenerative diseases, emphasizing the need of an efficient degradation system. An initiation of immunoproteasome expression is likely to be part of a cellular strategy to reduce the buildup of oxidatively damaged proteins in the cell under acute stress [145]. In agreement with this, reactive astrocytes and microglia surrounding amyloid beta plaques in aged AD mice display increased immunoproteasome expression and activity [7][chapter 2]. An initial activation of astrocytes in response to various stimuli might be crucial for protection against immediate damage. However, chronic reactivity of astrocytes, as observed in AD mice and in AD human brain tissue, is believed to induce astrocyte dysfunction contributing to a toxic environment for neurons (Orre et al., submitted [chapter 5]); [50]. In agreement with this hypothesis, lack of GFAP and vimentin has been shown to improve posttraumatic regeneration possibly via a change in cell signaling in astrocytes [211, 212]. In this respect, inhibition of chronic GFAP upregulation may be an attractive strategy to limit chronic astrocyte reactivity. Here, we demonstrate that blocking the induction of the immunoproteasome activity, using a specific β5i inhibitor, is sufficient to prevent an upregulation of GFAPα in astrocytes. β5i inhibition also reduced expression of pro-inflammatory molecules in LPS stimulated microglia isolated from AD mice [7][chapter 2]. Thus targeting activated astrocytes and microglia by immunoproteasome inhibition is likely to limit the secretion of pro-inflammatory cytokines as well as reducing the elevated GFAP expression, respectively. Hence, inhibition of the immunoproteasome represents a promising approach to prevent neuroinflammation without affecting the constitutive proteasome activity in neurons, needed to prevent toxic protein build-up.

However, astrocyte reactivity needs to be considered as “two edged”. In contrast to what is discussed above, in vitro activation of astrocytes using protea-
some activators induced an anti-inflammatory astrocyte phenotype in conjugation with an increased GFAP expression. Such phenotype differs from that of reactive astrocytes observed in AD mice, and emphasize that astrocytes with increased GFAP levels, commonly classified as “reactive”, are in fact heterogeneous ranging from a neurosupportive phenotype, to a proinflammatory, dysfunctional phenotype [47, 50]. Whether the regulation of GFAP differs between the two these phenotypes remains unclear, although STAT3 activation has mainly been implicated in neurotropic astrogliosis [202]. However, a transcription factor analysis on genes regulated in AD astrocytes show that STAT3 is predicted to be activated in chronically activated astrocytes while AP-1, c-fos or, c-jun appear unaffected (Orre et al, unpublished observations). Therefore, STAT3 activation appears to be involved in both neurotropic and pro-inflammatory forms of astrogliosis. This data indicates that the timeframe of stimulation may also be an important factor for the phenotype differences in astrocyte reactivity.

Taken together, we here demonstrate that an increase in (immuno)proteasome activity, leads to activation of Notch signaling in astrocytes in vitro, which in turn induces the upregulation of GFAP expression. Inhibition of Notch signaling is sufficient to prevent an induction of GFAP expression, without affecting Tgfb levels. This finding pinpoints Notch as an upstream regulator of proteasome induced GFAP activation in astrocytes (Fig. 7B). Inhibition of the immunoproteasome is sufficient to block the activation of the Notch pathway and in turn leads to a decrease in GFAP expression. Thus, inhibiting the immunoproteasome might be an attractive approach to control reactivity of astrocytes and to prevent chronic astrocyte reactivity. On the other hand, an activation of the proteasome, using the activators identified in this study, may be beneficial as later treatment strategy to boost the degradation of oxidative proteins and prevent inflammatory tissue damage. The link between proteasome activation and inhibition and its relation to the spectrum of astrocyte reactivity calls for further research to define whether and when modulation of proteasome activity would be beneficial to modulate astrocyte function in disease.

**Supplemental data**

For supplemental data please go to [http://www.ellyhollab.eu/](http://www.ellyhollab.eu/).