RESEARCH ARTICLE

Inhibition of NF-κB signaling in IKKβ^{F/F};LysM Cre mice causes motor deficits but does not alter pathogenesis of Spinocerebellar ataxia type 1

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Abstract

Spinocerebellar Ataxia type 1 (SCA1) is a fatal neurodegenerative genetic disease that is characterized by pronounced neuronal loss and gliosis in the cerebellum. We have previously demonstrated microglial activation, measured as an increase in microglial density in cerebellar cortex and an increase in the production of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), in the cerebellum of the ATXN1[82Q] transgenic mouse model of SCA1. To examine the role of activated state of microglia in SCA1, we used a Cre-Lox approach with IKKβ^{F/F};LysM Cre mice intended to reduce inflammatory NF-κB signaling, selectively in microglia. ATXN1[82Q];IKKβ^{F/F};LysM Cre mice showed reduced cerebellar microglial density and production of TNFα compared to ATXN1[82Q] mice, yet reducing NF-κB did not ameliorate motor impairments and cerebellar cellular pathologies. Unexpectedly, at 12 weeks of age, control IKKβ^{F/F};LysM Cre mice showed motor deficits equal to ATXN1[82Q] mice that were dissociated from any obvious neurodegenerative changes in the cerebellum, but were rather associated with a developmental impairment that presented as a retention of climbing fiber synaptic terminals on the soma of Purkinje neurons. These results indicate that NF-κB signaling is required for increase in microglial numbers and TNF-α production in the cerebella of ATXN1[82Q] mouse model of SCA1. Furthermore, these results elucidate a novel role of canonical NF-κB signaling in pruning of surplus synapses on Purkinje neurons in the cerebellum during development.

Introduction

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant neurodegenerative disease caused by the abnormal expansion of CAG repeats in the coding region of Ataxin1 (ATXN1)
gene [1]. The CAG repeat is translated into an expanded polyglutamine (polyQ) track in the ATXN1 protein [2], which places SCA1 into the family of polyglutamine diseases that also includes SCA 2, 3, 6, 7, 17, Kennedy disease, Huntington’s disease, and dentatorubropallidoluysian atrophy [3]. More than 39 uninterrupted CAG repeats in the ATXN1 gene causes SCA1, with the initial symptoms of ataxia, defined by movement and balance deficits, normally presenting in the patient’s mid-thirties. Motor deficits progressively worsen until premature death, commonly due to pulmonary compromise, 10–20 years from the disease onset [4]. There is no disease modifying therapy or cure for SCA1.

In vivo magnetic resonance imaging (MRI) demonstrated severe atrophy of the cerebellum and brain stem [5], and proton magnetic resonance spectroscopy (1H MRS) revealed neurochemical alterations in the cerebellum indicative of neuronal dysfunction/neurodegeneration and gliosis both in SCA1 patients and mouse models [6][7]. Postmortem analysis supports a predominantly cerebellar pathology with severe loss of Purkinje neurons and gliosis in the cerebellar cortex [8]. These pathological changes in the cerebellum are the likely cause of motor deficits observed in patients. In addition, longitudinal imaging studies demonstrated that cerebellar gliosis and neuronal dysfunction/degeneration correlate well with the clinical progression of disease [9][10][6][7]. We previously demonstrated that cerebellar microglia show signs of activation, defined by an increase in microglial density and hypertrophy of the soma and processes [11], in the ATXN1[82Q] transgenic mouse model of the SCA1 [10]. Furthermore, we have found that microglial activation was associated with increase in the production of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNFα), and was detectable prior to the loss of Purkinje neurons and motor deficits [10].

Microglia are resident phagocytic and immune cells of the central nervous system (CNS) that constitute about 5–12% of the brain glia [12]. Microglia are derived from the primitive macrophages in the yolk sac that colonize the neuroepithelium at E9.5 [13]. Microglia have significant roles in neuronal development [14][15][16][17][18], control of homeostasis of the healthy adult brain [19] and have been shown to have profound functions in most neurodegenerative diseases [20][21][22]. During development microglia play an important role in inducing and engulfing apoptotic neurons and glia as well as pruning immature surplus synapses to shape neuronal connectivity. However, while recent study demonstrated presence of phagocytic cups in microglia of developing rat cerebellum [18], little is known about their role in cerebellar development.

Upon sensing a change in brain homeostasis during injury or disease, microglia undergo morphological and functional change termed activation. While microglial activation is diverse and depends on the context of the insult and brain region, it often includes increase in microglial density, enlargement of cell bodies, and release of inflammatory cytokines that can modulate functions of astrocytes and neurons, such as brain-derived neurotrophic factor (BDNF) and TNFα [23][24][25][26][27][28][29][30][31]. While microglial activation has been described in many neurodegenerative disease, its functional role remains unclear as both beneficial and harmful effects have been reported [32][33][22][34].

Putative beneficial functions of reactive microglia can be accomplished through secretion neurotrophic factors such as BDNF and through the active removal of dying cells, non-functional synapses and extracellular aggregates via phagocytosis [22]. Activated microglia can be harmful by inappropriately removing functional synapses and thereby damaging the neuronal networks, and by inducing potentially neurotoxic inflammation. The microglia-induced neuroinflammation is largely mediated by the release of cytokines, including TNFα that have been shown to worsen outcome in various neurological disorders, including Alzheimer’s disease (AD), Multiple Sclerosis (MS), and Parkinson’s disease (PD) [35][36][37][38]. Microglial inflammation is modulated by the activity of the transcriptional factor nuclear factor kappa
(NF-κB), a master regulator of inflammation and a well-known regulator of TNFα expression [39]. Under normal physiological conditions, most of NF-κB is kept in the cytoplasm by being bound to the inhibitor of κB (IκB) proteins, therefore limiting NF-κB’s transcriptional activity [40]. Inflammatory stimuli commonly activate NF-κB through the classical inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) pathway, in which inducing stimuli trigger phosphorylation of catalytic unit of IκB kinase (IKK) complex, IKKβ subunit at Ser 180 [41] [42]. Activated IKKβ in turn phosphorylates IκB proteins leading to their ubiquitination and degradation [39], thus releasing NF-κB dimers to translocate to the nucleus and promote transcription of target genes. Compared to other brain regions, under physiological conditions cerebellar microglia exhibit a more alert immune phenotype that is in part regulated by NF-κB proteins [43]. However, how the NF-κB pathway in cerebellar microglia contributes to the pathogenesis of SCA1 is unknown.

Here, we used Cre-Lox approach to inhibit NF-κB signaling in microglia of SCA1 mice. We crossed IKKβF/F mice [44] with LysM Cre mice [45][44][46] to cause microglia selective deletion of IKKβ and transgenic mouse model of SCA1, ATXN1[82Q] mice that express mutant ATXN1 with 82 glutamine repeats only in Purkinje neurons (S1 Fig) [47]. We have found that ATXN1[82Q];IKKβF/F;LysM Cre mice had decreased density of microglia and expression of TNFα compared to ATXN1[82Q] littermates, suggesting that NF-κB pathway regulates early microglial recruitment and TNFα production in cerebella of SCA1 mice. However, onset and severity of SCA1 pathogenesis were not significantly altered in ATXN1[82Q];IKKβF/F;LysM Cre mice suggesting that early microglial neuroinflammation may not contribute to the pathogenesis of SCA1. This interpretation was complicated as we have also found that control IKKβF/F; LysM Cre mice exhibited motor deficits at 3 months of age associated with a reduction in developmental pruning of surplus synapses on soma of Purkinje neurons. Moreover, using reporter mice to examine Cre expression, we have found that despite LysM Cre line being previously used as a microglia specific in the CNS, Cre activity was also evident in cerebellar neurons, including Purkinje neurons.

**Results**

**NF-κB pathway contributes to increase in microglial density and TNF-α production in SCA1 mice**

We used a mouse genetic approach to reduce activation of the pro-inflammatory transcriptional regulator NF-κB selectively in microglia of SCA1 mice. The classical pathway of NF-κB signaling involves activation of IκB kinase (IKKβ) that phosphorylates IκB proteins marking them for ubiquitination and degradation. This frees NF-κB to translocate into the nucleus and mediate gene expression. We deleted the catalytic subunit of IKKβ using a Cre-LoxP approach and produced the IKKβF/F;LysM Cre mouse line, in which the putative microglia and macrophage specific Lysosome M promoter drives the expression of Cre-recombinase [45][48] to delete the exon 3 of IKKβ [44][49](S1 Fig). These mice were crossed with Purkinje neuron specific transgenic mouse model of SCA1, ATXN1[82Q] mice. ATXN1[82Q] mice demonstrate many features of SCA1 including increase in the cerebellar microglial density at four weeks of age and motor deficits and cerebellar pathology at 12 weeks [10].

We examined the effect of NF-κB inhibition on microglial density and morphology by performing immunohistochemistry for microglial marker ionic calcium binding protein (Iba1) (Fig 1A). As Cre recombinase is expressed throughout the brain in LysM Cre line it could potentially affect microglia in other brain regions involved in movement control, such as striatum. Thus, we first quantified microglia in the striatum of control and IKKβF/F;LysM Cre mice to control for these extra-cerebellar changes. We found that microglial density and
Inhibition of NF-κB is sufficient to cause motor deficits in IKKβF/F;LysM Cre mice but does not alter SCA1 motor abnormalities

We next determined whether reducing microglial NF-κB signaling affects motor deficits characteristic for SCA1. One of the earliest symptoms of SCA1 is ataxia, which refers to a loss of motor control and balance. Ataxia in mice can be quantified by the performance on the accelerating rotating rod (rotarod) test [51], where latency to fall of the rotarod is measured. Mice with cerebellar deficits, such as SCA1 mice, classically have reduced latency to fall due to impaired balance [50]. At three months of age, the earliest age at which we can detect motor deficits, we found that ATXN1[82Q];IKKβF/F;LysM Cre mice did not perform differently when compared to ATXN1[82Q] mice (Fig 2, ATXN1[82Q] mice 198s, N = 10, p > 0.05, one-way ANOVA with Bonferroni post-hoc testing).

Surprisingly, IKKβF/F;LysM Cre mice were significantly impaired in rotarod performance when compared to control mice (Fig 2, WT mice 370s N = 11, IKKβF/F;LysM Cre 198s, N = 10, p = 0.005, one-way ANOVA with Bonferroni post-hoc test). Moreover, haploinsufficient IKKβF/WT;LysM Cre mice that carry only a single floxed IKKβ allele also demonstrated motor deficits (S3 Fig). These results suggest that reducing canonical NF-κB signaling in IKKβF/F;LysM Cre mice is sufficient to cause motor impairments, but does not significantly affect SCA1-induced motor deficits.

We next determined whether motor deficits in IKKβF/F;LysM Cre are underlined by neuro-degenerative changes in Purkinje neurons, such as atrophy of soma and dendrites previously described in SCA1 mice [52][53]. We used immunofluorescence of parasagittal cerebellar...
slices with antibody against calbindin, a marker of Purkinje neurons that labels their cell bodies and processes (Fig 3A). In SCA1 mice Purkinje neuron atrophy can be quantified as a decrease in the calbindin intensity and a decrease in the width of the molecular layer [54]. Neither the molecular layer width nor the intensity of calbindin staining were altered in IKKβ\textsuperscript{F/F}; LysM Cre mice when compared to control WT mice (Figs 3B and 3C). We found a reduction in the width of the molecular layer in ATXN1\textsuperscript{[82Q]} mice compared to WT mice; yet this decrease was not significantly altered in ATXN1\textsuperscript{[82Q]}; IKKβ\textsuperscript{F/F}; LysM Cre mice (Fig 3B, normalized to N = 5 WT littermate controls IKKβ\textsuperscript{F/F}; LysM Cre mice 0.96 ± 0.07, N = 5, ATXN1\textsuperscript{[82Q]} mice 0.706 ± 0.12, N = 5, ATXN1\textsuperscript{[82Q]}; IKKβ\textsuperscript{F/F}; LysM Cre mice 0.68 ± 0.1, N = 5). Similar results were obtained for the intensity of calbindin staining that was decreased in both ATXN1\textsuperscript{[82Q]} and ATXN1\textsuperscript{[82Q]}; IKKβ\textsuperscript{F/F}; LysM Cre mice (Fig 3C, normalized to N = 5 WT littermate controls IKKβ\textsuperscript{F/F}; LysM Cre mice 0.96 ± 0.07, N = 5, ATXN1\textsuperscript{[82Q]} mice 0.706 ± 0.12, N = 5, ATXN1\textsuperscript{[82Q]}; IKKβ\textsuperscript{F/F}; LysM Cre mice 0.68 ± 0.1, N = 5).
Role of cerebellar microglia in development and disease

A recent study identified a set of genes that have reduced expression in Purkinje neurons in SCA1 mice that correlates well with disease progression [51]. Therefore, we examined the expression of these markers of Purkinje cell degeneration in cerebellar extracts from each of our experimental mouse lines using qRT-PCR. We have found a decrease in the expression of these genes in ATXN1[82Q] mice, as would be expected based on the published data (Fig 3D) [51]. However, expression of these genes was indistinguishable in ATXN1[82Q];IKKβF/F;LysM Cre and ATXN1[82Q] mice. Importantly, IKKβF/F; LysM Cre mice also showed a slight but not significant decrease in gene expression compared to wild-type littermates (Fig 3D, for example expression of Homer3 normalized to N = 5 WT littermate controls in IKKβF/F;LysM Cre mice 0.896 ± 0.01, N = 5, ATXN1[82Q] mice 0.465 ± 0.06, N = 5, ATXN1[82Q]; IKKβF/F; LysM Cre mice 0.485 ± 0.07, N = 5, and for Regulator of G protein signaling (Rgs8) normalized to N = 5 WT littermate controls IKKβF/F; LysM Cre mice 0.96 ± 0.11, N = 5, ATXN1[82Q] mice 0.41 ± 0.05, N = 5, ATXN1[82Q]; IKKβF/F;LysM Cre mice 0.49 ± 0.07, N = 5).

The expression of ataxin-1 or the marker of post-synaptic area post-synaptic density 95 (PSD95) proteins was also not changed in ATXN1[82Q];IKKβF/F;LysM Cre mice compared to ATXN1[82Q] mice (S4 Fig)[50]. Thus, our results indicate that reducing microglial NF-κB activity in ATXN1[82Q];IKKβF/F;LysM Cre mice is not sufficient to alter behavioral and neurological pathology in SCA1 mice, and that motor deficits in IKKβF/F; LysM Cre mice do not seem to be associated with degenerative changes in Purkinje neurons.

Cerebellar astrogliosis is not altered in ATXN1[82Q];IKKβF/F;LysM Cre mice

Astrocytes are another type of glial cells that are activated alongside microglia in brain injury [55][56]. Moreover, increasing evidence point to the importance of astrocyte-microglia communication in the pathogenesis of neurodegenerative diseases [28][57]. While we have found that both astrocytes and microglia are activated early in disease, it is unclear whether microglial NF-κB signaling is required or contributes to astrogliosis in SCA1 mice [10].

Therefore, we next examined whether cerebellar astrogliosis is altered in ATXN1[82Q]; IKKβF/F; LysM Cre mice. Astrogliosis was examined using immunohistochemistry of parasagittal cerebellar slices with the antibody against the marker of astrogliosis, glial fibrillary acidic protein (GFAP) (Fig 4A) [10]. As we have previously demonstrated there was an increase in the intensity of GFAP staining in the cerebella of ATXN1[82Q] mice compared to their wild-type littermates, that was not significantly altered in ATXN1[82Q]; IKKβF/F; LysM Cre mice. Moreover, IKKβF/F; LysM Cre mice were not different from control WT mice (Fig 4B, normalized to N = 6 WT littermate controls IKKβF/F; LysM Cre mice 1.04 ± 0.17, N = 6, ATXN1[82Q] mice 1.33 ± 0.18, N = 6, ATXN1[82Q]; IKKβF/F; LysM Cre mice 1.39 ± 0.27, N = 6). Classically activated microglia have recently been found to induce expression of neurotoxic astroglial A1 phenotype [58]. However, we have found only a trend towards lower expression of A1 astroglial genes, such as decreased expression of complement C3 (ATXN1[82Q] mice 1.78 ± 0.28, N = 4, ATXN1[82Q]; IKKβF/F; LysM Cre mice 1.48 ± 0.21, N = 4 normalized to N = 4 WT littermate controls, data not shown, P > 0.05 one-way Kruskal-Wallis test with Dunn’s test).
Purkinje neuron somatic synapse puncta are retained in IKKβF/F;LysM Cre mice

While IKKβF/F;LysM Cre mice demonstrate profound motor deficits on rotarod, we did not detect any overt degeneration in Purkinje neurons or activation of cerebellar astroglia (Fig 2, S3 Fig). Since pharmacological depletion of microglia in adult mice, did not cause any motor deficits [50], we reasoned that motor deficits seen in IKKβF/F;LysM Cre mice may be due to a developmental effect. To establish the appropriate synaptic connections during brain development it is critical to balance synaptogenesis, synapse pruning and maturation. Microglia are critical during this developmental time period where they serve to remove apoptotic cells and unnecessary synapses in order to establish functional neural circuits [17][59]. In cerebellum, shortly after birth, there are multiple climbing fiber (CF) synapses on the soma of Purkinje neurons that are removed during postnatal cerebellar development from P7-P21 [60][61]. Thus, in adult cerebellum approximately only one CF contacts proximal dendrites but not the soma of Purkinje neurons [62][63]. Furthermore, previous studies have shown that reduced synaptic pruning during development and the persistence of somatic puncta on Purkinje neurons impairs motor coordination in mice [64][62].

Since LysM promoter is active from E7 [45] it has potential to alter NF-κB signaling and thereby microglial function in synaptic pruning during development. We therefore tested whether there is a retention of somatic puncta in IKKβF/F;LysM Cre mice that could underline the found motor dysfunction. To quantify Purkinje neuron’s somatic synapse puncta, we co-stained cerebellar slices with calbindin and a marker of climbing fiber terminals vesicular glutamate transporter 2 (VGLUT2). By using in house written ImageJ macro to objectively quantify the number of somatic puncta, we found that IKKβF/F;LysM Cre mice had significantly more unpruned CF terminal puncta on the soma of Purkinje neurons than WT mice (Fig 5, WT 4.5 ± 0.6, N = 3, IKKβF/F;LysM Cre mice 10.12 ± 0.24, N = 4, student’s t-test, P = 0.0156). We have found similar results in IKKBWT;LysM Cre mice (S5 Fig). This result may suggest that the canonical NF-κB pathway in microglia has important role in pruning somatic synapses on Purkinje neurons during cerebellar development.

LysM Cre is not selective for microglia

To test the specificity and efficiency of Cre activation in microglia, we crossed LysM Cre mice with the reporter GCaMP6 mice [65]. These reporter mice have a floxed-STOP cassette preventing transcription of the GCaMP6 (that consists a green fluorescent protein GFP fused to a calcium binding calmodulin and M13 protein domains). Cre recombinase in LysM cells should excise STOP cassette thereby leading to GCaMP6 expression and GFP immunoreactivity. Cerebellar slices from GCaMP6;LysM Cre mice were stained against GFP (green) and microglial maker Iba1 (Fig 6A). Only around 10–15% of cerebellar microglia were co-labeled with GFP and Iba1. This low microglial efficiency of LysM Cre line was previously demonstrated [44] and may contribute to the lack of changes in SCA1 severity in ATXN1[82Q]; IKKβF/F;LysM Cre mice. Moreover, we were surprised to detect GFP positive cells in the molecular and Purkinje cell layers that were co-labeled with parvalbumin (PV), a marker of GABA-ergic interneurons. We found that approximately 20% of GFP positive cells in the molecular layer are also PV-positive interneurons and Purkinje cells (Fig 6B). We tried to confirm these
results with IKKβ immunohistochemistry of cerebellar slices, but we could not achieve good staining with several commercially available anti-IKKβ antibodies. Thus, it is possible that reduced pruning and motor deficits in \(IKKβ^{F/F};\text{LysM Cre}\) mice may be caused by loss of IKKβ in cerebellar neurons.

**Discussion**

We have found that \(ATXN1[82Q];IKKβ^{F/F};\text{LysM Cre}\) mice have reduced microglial density and TNFα expression in cerebellum, but undistinguishable motor and cerebellar neurological...
phenotype compared to ATXN1[82Q] mice. Moreover, we have found motor deficits in IKKβF/F;LysM Cre mice that were dissociated from overt Purkinje cell degeneration but instead, associated with abnormal retention of climbing fiber synapses on Purkinje soma. These results may indicate that microglial NF-κB signaling is important for pruning of immature surplus synapses during cerebellar development, but it is not critical to the pathogenesis of SCA1.

Microglia are the resident phagocytic cells of the brain that derive from primitive myeloid progenitors in yolk sac and populate the brain during early embryonic development. As such, microglia are well positioned to influence brain development and sculpt functional neuronal connections, including elimination of synapses and neurons. Pruning of immature surplus synapses is critical for the establishment of the appropriate synaptic connections during brain development [17] [59]. In cerebellum, shortly after birth, there are multiple climbing fibers (CFs) that contact Purkinje neuron soma, while in adult cerebellum one climbing fiber contacts proximal dendrites but not soma of Purkinje neurons [55]. This change in wiring is achieved during postnatal days (P) 7–21 when one “winning” CF translocate synapses to the proximal dendrites of Purkinje neurons while CF synapses on the soma of Purkinje neurons are pruned [63]. Since previous studies have shown that reduced synaptic pruning during development and ensuing persistence of somatic puncta on Purkinje neurons can impair motor coordination in mice [64] [62], it is possible that observed persisting somatic CF synapses in Purkinje neurons in adult IKKβF/F;LysM Cre mice could contribute to motor deficits exhibited by these mice. While role of microglia in the cerebellar development is not well understood [18], our novel results may suggest that the canonical NF-κB pathway in microglia, typically considered as pro-inflammatory, is required for the removal of immature synapses during cerebellar development.

In chronic disease microglia are thought to be neurotoxic in part due to the increased production of pro-inflammatory cytokine TNFα. TNFα is one of the key microglial cytokines that modulates synaptic strength in response to neuronal activity in physiological conditions [66]. As different levels of TNFα are known to increase or decrease synaptic strength, regulation of its expression in microglia is tightly regulated, in part through NF-κB signaling [67] [68]. In our previous experiments we have demonstrated that microglial density is increased presymptomatically in SCA1 mice concurrent with increased production of TNFα [10]. Moreover, we have found that pharmacologically depleting microglia early in SCA1 reduced production of TNFα [50], indicating that microglia produce most of TNFα in SCA1 mice. However, despite the observed decrease in microglial density and expression of TNFα, SCA1 motor and neurological pathology were not ameliorated in ATXN1[82Q];IKKβF/F;LysM Cre mice. While these results may suggest that microglia and TNFα expression are not critical for the pathogenesis of SCA1 during early stages of disease, it is important to note that neither microglial density nor TNFα expression were fully reversed.

One likely reason for the partial reduction of neuroinflammation and the lack of differences between ATXN1[82Q] and ATXN1[82Q];IKKβF/F;LysM Cre mice is the low efficiency of LysM Cre line. Using reporter mice we have found that approximately 15% of cerebellar microglia showed Cre activity as was previously demonstrated [44]. Thereby, it is possible that inhibition of microglial NF-κB pathway is beneficial in SCA1, but that due to the low efficiency of Cre recombination in LysM Cre line, we did not reach the threshold of NF-κB inhibition necessary for the significant alteration of disease phenotype.

However, it is important to note that this low efficiency of LysM Cre line was sufficient to modulate pathogenesis in mouse models of several conditions, including AD [48], Rett syndrome [46], multiple sclerosis [70], ischemia [44], obesity [71], and depression [72]. For example, genetic deletion of IKKβ in microglia ameliorated neuronal loss in a mouse model of excitotoxicity and ischemic brain injury [44]. LysM Cre deletion of IKKβ in the microglia of
mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) mice, delayed the onset and alleviated the severity of EAE neurological symptoms, such as ataxia and paralysis of limbs [73]. Furthermore, LysM Cre mice were used to demonstrate that microglial progranulin deficiency increases plaque deposition and impairs phagocytosis in AD [74] and that targeted expression of MECP2 in myeloid cells, driven by LysM Cre promoter in an Mecp2-null background attenuated pathology in a mouse model of Rett syndrome [46]. Thus, because low efficiency of LysM Cre had a significant effect in these other diseases, it is also possible the role of microglia in pathogenesis may be specific to each disease or to the stage of disease [75]. For example Liao et al. demonstrated that in mouse model of ALS microglia have neuroprotective role at disease onset, whereas at the end-stage of disease microglia have a neurotoxic role, supporting the transformation of microglial phenotype during disease progression [75].

In this study we have focused on the early stages on SCA1, due to their significant therapeutic potential. It is possible that role of microglial neuroinflammation in the pathogenesis of SCA1 may change as the extent of injury worsens with disease progression. Future studies testing the role of microglial NF-κB signaling in the late stage of SCA1 using inducible Cre-LoxP system are needed to address this question. However, cerebellar microglia are not well studied and it is also possible that cerebellar microglia may play a different role in disease than microglia in other brain regions. Genome-wide analysis of microglia from different brain regions demonstrated that cerebellar microglia exist in a more immune-vigilant state that is further augmented during aging [43]. Time-lapse in vivo imaging, found that cerebellar microglia have decreased parenchymal surveillance compared to cortical microglia [76]. Thus our data may also indicate that cerebellar microglia and in particular NF-κB signaling behave differently in disease compared to microglia from other brain regions.

Another possible explanation for the observed lack of amelioration of disease phenotype in ATXN1[82Q];IKKβF/F;LysM Cre mice is that reactive astrocytes have a predominant role in contributing to the pathogenesis of SCA1. Astrocytes have numerous important functions in the brain, including structural and metabolic support of neurons, and regulation of extracellular ion and neurotransmitter homeostasis [77][78]. Similar to microglia, astrocytes also undergo activation in neurological diseases that results in morphological and functional changes, including enlarged cell bodies, thickening of cell processes, and alteration in their ability to maintain homeostasis and to provide neurotrophic support. Liddelow et al. demonstrated that LPS activated microglia induce neurotoxic phenotype of astroglia though the secretion of pro-inflammatory cytokines, including TNFα [58]. We have not detected a significant alteration of astrogliosis, measured as increased expression of GFAP and A1-associated genes in ATXN1[82Q];IKKβF/F;LysM Cre cerebella, indicating that reduced microglial density and TNFα expression may not be sufficient to affect astrogliosis and its possible neurotoxic effects in SCA1 mice.

Finally, when interpreting disease phenotype in ATXN1[82Q]; IKKβF/F;LysM Cre mice we need to consider specificity of the LysM Cre line [45][79][80][74]. We examined the cerebellar cell specificity of the LysM Cre line, by crossing them with the reporter line [65], and were surprised to find evidence of Cre recombination not only in microglia as expected, but also in neuronal cells including Purkinje neurons and parvalbumin (PV) positive interneurons. Thus, it is possible that a decrease in microglial NF-κB and neuroinflammation in the cerebellum may have a beneficial effect on SCA1 pathogenesis, but this is masked by a negative effects of decreased NF-κB in Purkinje neurons [81]. Similarly, while the observed retention of climbing fiber puncta on the soma of Purkinje neurons is sufficient to cause motor deficits in IKKβF/F; LysM Cre mice, it is possible that decreased NF-κB activity in Purkinje neurons contributes to these motor deficits.
In addition, while efficiency of LysM Cre mediated deletion may be higher in the cerebellum compared to the other regions of the brain [82], it is by no means limited to the cerebellum. Thus, it is possible that loss of IKKβ in other brain regions contributes to the impaired motor phenotype of IKKβ; LysM Cre mice. We have not found any change in microglial density or morphology in the striatum, a brain region involved in the control of movement, but we cannot exclude subtle changes in microglial function. To resolve these issues of cell and region specificity of IKKβ depletion future studies using more selective Cre lines or AAV viruses expressing Cre recombinase to selectively target cerebellar microglia, Purkinje neurons or PV interneurons are needed. Moreover, previous studies, including the comprehensive evaluation of several myeloid-expressing Cre strains, have demonstrated LysM-Cre mediated deletion in macrophages, neutrophils, and monocytes [83][84]. Thus, while depletion of IKKβ in microglia may be beneficial in the context of SCA1 (e.g. by lowering neuroinflammation), it is possible that loss of IKKβ in these other myeloid cells may negatively impact on the motor function in mice and thereby contribute to the unaltered disease severity in ATXN1[82Q]; IKKβF/F; LysM Cre mice or to the motor deficits in IKKβF/F; LysM Cre mice. Importantly our study strongly cautions against using LysM Cre mice to study microglia specific effects and calls for considering neuronal and myeloid cell recombination when re-interpreting previous CNS studies using these mice.

Materials and methods

Mouse lines
ATXN1[82Q], IKKβF/F and LysM Cre mice were generated as previously described [45][71][47]. Because our previous studies have detected no sex-specific effects in SCA1, we have used an equal mix of animals of both sexes for our experiments. All animal experiments were performed in compliance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals and the University of Minnesota Institutional Animal Care and Use Committee.

The protocol was approved by the University of Minnesota Institutional Animal Care and Use Committee (protocol number 1511-33160A).

Rotating rod test
Motor deficits were assessed using rotarod assay at the age of 3 months. The rotarod test was performed as previously described [85]. Briefly, mice were placed on the rotarod apparatus (Ugo Basile) that accelerates from a speed of 4 rotations per minute (rpm) to 40 rpm over a 5-minute period. We recorded the time it takes for a mouse to fall off rotarod, to a maximum of 10 minutes. Mice were subjected to four trials per day for four consecutive days, with at least ten minutes of rest between each trial. Data for the performance on day 4 was analyzed using one-way ANOVA with Bonferroni post-hoc test. Significance was assumed at P < 0.05. All tests were performed blinded with respect to the genotype.

Immunohistochemistry
Mouse brains were fixed overnight in 4% formaldehyde, incubated in 30% sucrose, and cut into 45 µm sections on cryostat (Leica, CM 1850). Sections were washed three times in cold Phosphate Buffered Saline (PBS), and incubated in blocking buffer (3% Normal Donkey Serum in PBST (1% Triton X-100 PBS) for 1 hr. at room temperature (RT). This was followed by an overnight incubation at 4°C in blocking buffer containing the relevant primary antibody [anti-GFAP (Z0334, DAKO), anti-Iba1 (019–19741, WAKO), anti-Calbindin-D-28K (C9848, Sigma Aldrich), anti-VGLUT2 (MAB5504, Millipore), anti-ataxin1 (11NQ, a gift from Dr.
Harry Orr). After incubation with primary antibody we washed samples three times with PBS, and incubated them overnight at 4°C with the relevant fluorescently labeled secondary antibody (Alexa, Invitrogen). We mounted stained sections on slides with Vectashield mounting media containing DAPI (Vector Laboratories) for imaging at 20X magnification under the confocal microscope (Olympus FV1000). For each mouse, we imaged at least six different, randomly selected cerebellar lobules. Each image consisted of 20 μm Z-stacks, composed of 1μm thick image slices. Images were analyzed using FIJI (ImageJ, NIH) software. For analysis of calbindin length and intensity, we drew a straight line extending from the middle of Purkinje cell body to the end of the molecular layer in at least two places for each lobule. We then used the Measure function in ImageJ to quantify the length of the line to obtain measurement of the width of the molecular layer, and the average intensity along the line to obtain calbindin intensity. For GFAP staining, we used the Measure function in ImageJ to quantify the intensity of signal in the molecular layer that was then normalized to the average intensity in the control group (wild-type mice). For microglial Iba1 staining, we counted the Iba1 positive cells and to obtain microglial density we divided the number of Iba1 positive cells by the area in which they were counted. Staining, microscopy, and image analysis were performed blinded to the experimental groups. Data was analyzed using ANOVA with Bonferroni post-hoc test using GraphPad Prism software.

For counting puncta, tissue was stained in the same manner using antibodies against calbindin and VGLUT2 and then imaged at 60X magnification (20 μm Z stack, 1 μm Z step). Images were then processed using an in-house written ImageJ macro that would segment the calbindin and VGLUT2 signals, and procedurally count VGLUT2 puncta (minimum size 1.4 μm², maximum μm³ 70.3 size filter for puncta) on somata of Purkinje cells, defined by volume segmentation (minimum/maximum volume 281.3 and 7,031.3 μm³ respectively). Resultant images of segmented somata and counts were filtered by hand to remove artefactual somata. Counts were compared using a student’s t test.

**Quantitative real time RT-PCR**

Total RNA was isolated from cerebella using TRIzol (Invitrogen) and treated with DNase (TURBO Dnase, Thermo Fisher Scientific). Complementary DNA (cDNA) synthesis was performed in duplicate, using Superscript III First-Strand Synthesis SuperMix (Invitrogen) and random hexamer primers. The expression level of each gene was determined on a Light Cycler 480 II (Roche) using Light Cycler 480 SYBR Green PCR I Master mix (Roche). Cycling conditions were: 5 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 56°C for 1 min. Samples were analyzed in triplicate and a melting curve analysis was performed in each sample at the end of the qPCR reaction to confirm specificity of reaction. Expression levels of mouse 18S RNA (forward primer: 5’AGT CCC TGC CCT TTG TAC ACA 3’ and reverse primer: 5’ CGA TCC GAG GGC CTC ACT A -3’) were used as internal controls. Primers used for the Purkinje neuron’s Magenta cluster genes (*calbindin*, *ITPR*, *INPP5a*, *Garnl3*, *Pcp4*, *Homer3* and *Rgs8*) were from Ingram et al. [51], and primers used for astroglial genes (*Kir4.1, EAAT1, P2RY, Aqp4, C3, S100*, and *Glul*) and microglial gene (*TNFα*) were PrimeTime qPCR primers (IDT). Relative gene expression was determined by the 2^ΔΔCt method [86]. The threshold cycle (Ct) value was determined for target genes and the endogenous internal controls (18S RNA) in each sample. The difference between target gene and 18S RNA control Ct values was determined for each sample, resulting in the ΔCt value. The ΔCt of a calibrator, a wild type sample, was subtracted from each sample’s ΔCt to yield the ΔΔCt value. Relative fold change was calculated as 2^ΔΔCt. Data was analyzed using ANOVA with Bonferroni post-hoc test using GraphPad Prism software.
Western blotting

Cerebella were dissected from mice and lysed in RIPA lysis buffer [50mM Tris HCl, pH7.4, 150mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.2% SDS, phosphatase (Sigma) and protease inhibitors cocktail (Roche)]. After three cycles of freeze and thaw, proteins were separated on a 12% or 15% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The following primary antibodies were used: anti-ATXN1 (rabbit 11NQ, Orr lab), anti-PSD95 (Biolegend), and alpha-tubulin (mouse, Sigma). Signals from secondary antibodies linked to horseradish peroxidase (HRP) (GE Healthcare) were detected using Amersham ECL Western Blotting Detection Reagent (GE Healthcare) and ImageQuant LAS 4000 imager (GE Healthcare); protein levels were quantified using ImageQuant (GE healthcare) and ImageJ. Data was analyzed with one-way ANOVA followed by Bonferroni post-hoc test.

Statistical analysis

Statistical tests were performed with GraphPad Prism or R. For rotarod, we have used one-way ANOVA followed by either Tukey’s HSD or Bonferroni post-hoc test. For IHC, qRT-PCR, and Western blot quantification, we have also used Kruskal-Wallis test with Dunn’s test or one-way ANOVA followed by Bonferroni post-hoc test.

Supporting information

S1 Fig. A novel SCA1 transgenic mouse line ATXN1[82Q]; IKKβ<sup>F/F</sup>;LysM Cre was created to express Cre-recombinase specifically in microglia under the control of LysM promoter, and to remove loxP-enclosed exon 3 of IKKβ from microglia. (TIF)

S2 Fig. Microglial density in the striatum of IKKβ<sup>F/F</sup>;LysM Cre mice. A. Brain slices from IKKβ<sup>F/F</sup>;LysM Cre and control WT littermates were stained with Iba1 at three months of age. Insets show magnified images of microglia. B. Quantification of microglial density in the molecular layer (N ≥ 3 per each genotype), Student’s t-test P = 0.9008. Each dot represents one mouse, and values indicate mean ± SEM. (TIF)

S3 Fig. Motor function in IKKβ<sup>F/WT</sup>;LysM Cre mice. IKKβ<sup>F/WT</sup>;LysM Cre and control WT littermates were tested on a rotarod at three months of age. Each dot represents one mouse, and values indicate mean ± SEM, * indicates P < 0.05 by one-way ANOVA with Bonferroni post-hoc test. (TIF)

S4 Fig. Ataxin-1 and PSD 95 protein levels in ATXN1[82Q]; IKKβ<sup>F/F</sup>;LysM mice. Ataxin-1 (A) and PSD95 (B) protein levels were examined using western blotting of cerebellar lysates from 3-month-old mice. Each dot represents one mouse, and values indicate mean ± SEM, data was analyzed using one-way ANOVA followed by Bonferroni post-hoc test. (TIF)

S5 Fig. Reduced synaptic pruning in the cerebella of IKKβ<sup>F/WT</sup>; LysM Cre mice. A. Cerebellar tissue stained with Calbindin (red) and VGLUT2 (green). B. Average number of somatic puncta on Purkinje neurons. * Student’s t-test P < 0.05. (TIF)
Acknowledgments

We are grateful to Harry Orr for the generous gift of mouse lines and to all the members of Cvetanovic and Orr laboratories for suggestions.

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