Reversibility of symptoms in a conditional mouse model of spinocerebellar ataxia type 3

Jana Boy1, Thorsten Schmidt1, Hartwig Wolburg2, Andreas Mack3, Silke Nuber1, Martin Böttcher1, Ina Schmitt4, Carsten Holzmann5, Frank Zimmermann6, Antonio Servadio7 and Olaf Riess1,*

1Department of Medical Genetics, 2Institute for Pathology and 3Institute for Anatomy, University of Tuebingen, D-72076 Tuebingen, Germany, 4Clinic for Neurology, University of Bonn, D-53127 Bonn, Germany, 5Department of Medical Genetics, University of Rostock, D-18055 Rostock, Germany, 6Center for Molecular Biology, University of Heidelberg, D-69120 Heidelberg, Germany and 7San Raffaele Scientific Institute, I-20132 Milan, Italy

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Spinocerebellar ataxia type 3 (SCA3) is caused by the expansion of a CAG repeat tract that affects the MJD1 gene which encodes the ataxin-3 protein. In order to analyze whether symptoms caused by ataxin-3 with an expanded repeat are reversible in vivo, we generated a conditional mouse model of SCA3 using the Tet-Off system. We used a full-length human ataxin-3 cDNA with 77 repeats in order to generate the responder mouse line. After crossbreeding with a PrP promoter mouse line, double transgenic mice developed a progressive neurological phenotype characterized by neuronal dysfunction in the cerebellum, reduced anxiety, hyperactivity, impaired Rotarod performance and lower body weight gain. When ataxin-3 expression was turned off in symptomatic mice in an early disease state, the transgenic mice were indistinguishable from negative controls after 5 months of treatment. These results show that reducing the production of pathogenic ataxin-3 indeed may be a promising approach to treat SCA3, provided that such treatment is applied before irreversible damage has taken place and that it is continued for a sufficiently long time.

INTRODUCTION

Spinocerebellar ataxia type 3 (SCA3), also known as Machado–Joseph disease (MJD), is an autosomal dominantly inherited, neurodegenerative disorder caused by the expansion of a CAG repeat in the MJD1 gene that results in an expanded polyglutamine repeat in the encoded ataxin-3 protein (1). SCA3 belongs to the group of at least nine polyglutamine diseases, among them Spinocerebellar ataxia types 1, 2, 6, 7 and 17 as well as Huntington’s disease (HD), spinal bulbar muscular atrophy and dentatorubral-pallidoluysian atrophy (reviewed in 2). Whereas in unaffected persons the MJD1 gene (or ataxin-3 gene) contains up to 47 CAG repeats, in patients expanded repeats of up to 86 CAG are described (3). SCA3 is characterized clinically by a disturbance in the coordination of movements (cerebellar ataxia), bulbar, pyramidal and extrapyramidal symptoms as well as by peripheral neuropathy and ophthalmoplegia (4). In postmortem brains of SCA3 patients, degeneration and loss of neuronal cells in characteristic brain regions can be observed including the dentate nucleus of the cerebellum, the subthalamic nucleus, the globus pallidus and the red nucleus of the basal ganglia as well as the pontine nuclei (5). One hallmark of the disease is the formation of intranuclear inclusion bodies (6,7).

Previously generated mouse models of SCA3 (8–12) have allowed for the study of the progressive course of the disease and dissection of pathogenetic steps. These models, however, do not allow the analysis of whether a certain symptom, once manifested, is reversible.

Recently, the phenotype of mice expressing ataxin-3 exclusively in Purkinje cells was rescued by lentiviral-mediated expression of a guanosine triphosphatase facilitating the degradation of polyglutamine-containing aggregates (13). However, it is unclear whether this approach can also be applied to other brain regions or whether targeting ataxin-3 itself can be beneficial. Recently, it has been demonstrated that injections of
specific lentiviral vectors can silence expanded ataxin-3 in an allele-specific manner (14). However, by this technique only very restricted brain regions can be targeted.

Here we present the first inducible mouse model for SCA3 applying the Tet-Off system (15) under the control of a prion protein promoter (16), which leads to strong expression of the transgene in the cerebellum (17,18). Transgenic mice develop a phenotype resembling that of human patients of SCA3: the mice show a progressive neurological phenotype characterized by hyperactivity, reduced anxiety, impaired motor learning, weak Rotarod performance, reduced weight and intranuclear inclusion bodies. After turning off the expression of the expanded ataxin-3, however, the observed motor symptoms regress and after 5 months of treatment, transgenic SCA3 mice are indistinguishable from their control littermates. In our in vivo mouse model, we therefore demonstrate that both neurological and physiological symptoms of SCA3 are reversible.

RESULTS

Generation of a conditional SCA3 mouse model

We used the Tet-Off system developed by Gossen and Bujard (15) to generate a conditional mouse model of SCA3. We use a construct containing the full-length human ataxin-3c isoform (GenBank accession number: U64820), which contains an additional ubiquitin-interacting motif at its C terminus (17,18). One copy of the transgene containing ~70 CAG repeats is integrated in the generated responder lines 2909, 2776 and 2723. Line 2285 contains two copies, one containing ~30 CAG repeats and another with 66 repeats. The latter is shaded since no expression was detectable for this transgene copy. In responder line 2904, integration of three transgenes occurred: one copy with 60 CAG repeats, another with 65 and a third with ~80.
Brain tissue lysates of double transgenic mice from each line were analyzed for ataxin-3 transgene expression using western blot analysis. For all lines, expression of ataxin-3 was detected, but the sizes and the numbers of transgene bands varied between the different lines (Fig. 1B). Fragment analyses revealed that differences in the numbers of CAG repeats were responsible for the varying sizes of the ataxin-3 transgene bands (Fig. 1C). Three responder mouse lines contain one copy of the ataxin-3 transgene construct with 70 CAG repeats, whereas in two lines more than one copy is integrated (Fig. 1D). For SCA3 responder line 2285, two copies of the transgene with different CAG repeat lengths were detected, one containing about 30 units and the second 66 CAG repeats. On the protein level, however, only the short fragment (with ~30 repeats) was detectable by western blot analysis (Fig. 1B). Similar analyses of responder line 2904 detected three transgene copies with differing CAG repeat numbers. Two copies comprise about 60 CAG repeat units with small differences (~60 and ~65 CAG repeats) and one copy contains approximately 80 repeats (Fig. 1C and D). Following the CAG repeats over several generations revealed that the transmission of the two transgene copies in line 2285 and the transmission of the three copies in line 2904 are linked in each case, indicating one integration site for the respective transgene (data not shown). The integration of multiple copies of the ataxin-3 transgene was also confirmed by Southern blot analysis (Supplementary Material, Fig. S1).

SCA3 responder line 2904 expressed an ataxin-3 form with the highest number of CAG repeats, and it expressed this protein at high levels. These reasons, together with the fact that this mouse line bred successfully, led us to choose it for detailed behavioral and neuropathological characterization. In the following, double transgenic mice with the PrP promoter and the SCA3 responder (line 2904) are designated PrP/MJD77.

**Influence of the zygosity of the promoter or responder on transgene ataxin-3 level**

In some mouse models of SCA3, a disease phenotype is manifested only when the mice are homozygous for the ataxin-3 transgene (11). In general, higher expression levels lead to stronger and earlier symptoms in mice (10). To analyze whether the zygosity of the PrP promoter and/or the SCA3 responder transgene affects the expression level, we determined the zygosity by real-time PCR using hybridization probes and the 2 \(^{-\Delta Ct}\) method described by Livak and Schmittgen (22). The following terms for the different groups of double transgenic genotypes are used in this study:

- hetero/hetero—heterozygous for the PrP promoter and the ataxin-3 responder construct
- hetero/homo—heterozygous for the PrP promoter and homozygous for the ataxin-3 responder
- homo/hetero—homozygous for the PrP promoter and heterozygous for the ataxin-3 responder
- homo/homo—homozygous for both the PrP promoter and the ataxin-3 responder construct

In a conditional mouse model of SCA1, robust expression of the transgene was achieved only in mice homozygous for the responder construct (23). In our present study, we analyzed the zygosity of not only the responder construct but also the promoter construct. The influence of the zygosity of both the promoter and the responder has not been analyzed in any previous study of Tet-Off mouse models. We thus investigated by western blot whether differences in the expression level between different genotype groups do exist. Indeed, hetero/homo mice showed a slightly higher level of transgene protein, and homo/homo mice a much higher level of ataxin-3 transgene protein, than did hetero/hetero mice (Fig. 2).

**Expression pattern of ataxin-3 in double transgenic mice**

To study the distribution of ataxin-3 transgene expression in brains of double transgenic PrP/MJD77 mice, western blot analyses were performed. Brains were divided into four subregions (Fig. 3A). Whereas only a weak expression of human ataxin-3 was detected in the telencephalon, striatum and brainstem, a strong signal was seen in the cerebellum (Fig. 3B). However, in double transgenic PrP/MJD77 mice treated with doxycycline (dox) for a period of 3 weeks, no human ataxin-3 expression was detected by western blot analysis (Fig. 3B), indicating a complete block of transgenic ataxin-3 expression with no residual expression.

We then studied the expression pattern of PrP/MJD77 mice by immunohistochemistry. Paraffin-embedded sections were labeled with an antibody against ataxin-3, and immunoreactivity was observed in particular in the cortex, hippocampus, the brainstem and pons. The strongest signal, however, was detected in the cerebellum (Fig. 3C and D). These results are similar to the expression observed in a previous analysis of the PrP promoter (17). Immunohistochemical analyses of mice treated with dox for 2 weeks did not show any detectable transgenic ataxin-3 protein either (Fig. 3D).

To identify the cell types with the strongest transgene expression in the cerebellum, we performed double
Figure 3. In PrP/MJD77 mice, the transgene is expressed predominantly in the cerebellum, and the expression is completely blocked by doxycycline. (A) Schematic picture to show the subregional division of brain tissue for western blot analysis [adapted from Paxinos and Franklin (50)]. (B) Western blotting with an anti-ataxin-3 antibody gave the strongest ataxin-3 transgene signal (arrow) in the cerebellum (cer) of a PrP/MJD77 mouse (line 2904) without doxycycline treatment (−dox), and weaker signals were detected in the telencephalon (tel), the striatum (str) and the brainstem (brst). After treatment of a double transgenic mouse for 3 weeks with doxycycline (+dox), no more transgenic ataxin-3 protein was detected in the western blot analysis. β-Actin was used as a control for equivalent loading of the protein lysates. (C) Immunohistochemical staining of brain tissue of PrP/MJD77 mice. Low magnification pictures of four representative brain regions after staining with an anti-ataxin-3 antibody (1H9) showing ataxin-3 transgene expressing cells in several regions with the strongest signals in the cerebellum. (D) Higher magnification pictures of anti-ataxin-3 (1H9) stained brain sections again showing the expression pattern in several brain regions of a fully expressing PrP/MJD77 mouse (−dox). However, the intensity of the staining of a PrP/MJD77 mouse treated with doxycycline for 2 weeks (+dox) was comparable to that seen in a single transgenic control (control). Therefore, after doxycycline treatment, no more ataxin-3 transgene expression was detected immunohistochemically. Representative images of four of 20 mice analyzed by immunohistochemistry. Scale bars: (C) 100 μm; (D) 20 μm.
immunofluorescence staining of paraffin-embedded sections. Although no overlap with a neuron-specific antibody (NeuN) (Fig. 4A) or an anti-calbindin antibody (Fig. 4B) and the anti-ataxin-3 antibody (1H9) signal was observed, at least a partial co-localization was seen using an astrocyte-specific marker (glial fibrillary acidic protein, GFAP) (Fig. 4C). Given the shape and the localization, these data support the suggestion that theses cells correspond with Bergmann glia cells (U. Rüb, personal communication). Such a finding would be consistent with our previous analysis of the transgenic PrP promoter line (17).

**Aggregates and neuronal dysfunction**

As a hallmark of SCA3 and other polyglutamine diseases, intranuclear inclusion bodies form in neuronal cells of affected brain regions (6,7). In previously described SCA3 mouse models, such intranuclear inclusions have been observed (8,10–12). In the case of the conditional PrP/MJD77 model described here, intranuclear aggregates staining positive for ataxin-3 were detected in some neuronal cells of the cerebral cortex (Fig. 5), and the occurrence of such aggregates clearly correlated with the gene copy number. In brains of double transgenic PrP/MJD77 mice homozygous for at least one of the two transgenes, aggregates were already detectable in extremely young animals, e.g. at the age of 1 month in a hetero/homo mouse (Fig. 5A). In contrast, the first inclusions staining positive for ataxin-3 in heterozygous mice (hetero/hetero) became visible at the age of 20 months (Fig. 5D). This result indicates the utility of the inducible Tet-Off system for studying gene dose effects in neurodegeneration.

We next studied whether cell loss or degeneration can be observed in double transgenic mice. Electron microscopic analyses confirmed that Purkinje cells are damaged in 20-month-old mice (Fig. 6A). These analyses again revealed a gene dose effect: whereas mainly normal Purkinje cells were visible at the age of 20 months in brains of hetero/hetero mice and controls (Fig. 6A, I and II), Purkinje cell pathology was visible in age-matched double transgenic mice homozygous for either the promoter (Fig. 6A, III) or the responder construct (Fig. 6A, IV). Cells of this type appeared darkly stained (dark cell degeneration) due to higher electron density. Furthermore, the analyses of sections stained with an antibody directed against calbindin revealed a tendency towards smaller sizes of Purkinje cells as well as towards a reduced thickness of the cerebellar molecular layer in 21-month-old double transgenic mice homozygous for both transgenes (homo/homo) (Fig. 6B). Homozygous mice that were treated from the age of about 2

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Figure 4. Analysis of cell types expressing the ataxin-3 transgene in the cerebellum of PrP/MJD77 mice. Double staining with anti-ataxin-3 (7) and a neuronal cell-specific marker (NeuN) (A), as well as with the anti-ataxin3-antibody 1H9 and calbindin (B) did not result in colocalized fluorescence signals. Combined staining with an antibody against astrocytes (GFAP) and an anti-ataxin-3 antibody (1H9) (C), however, led to overlapping signals. These results demonstrate expression of the ataxin-3 transgene in glial cells (astrocytes) in the cerebellum. Abbreviations: GrCb, granular layer of the cerebellar cortex; MoCb, molecular layer of the cerebellar cortex; PuCb, Purkinje cell layer of the cerebellar cortex; wm, white matter. Representative images of one of four mice analyzed. Scale bar, 10 μm.
months on (homo/homo-dox), however, showed measures of Purkinje cell size and molecular layer thickness comparable to those seen in controls.

Behavioral phenotype and alteration of motor coordination

Double transgenic mice, especially those which were homozygous for one or both of the transgene constructs, displayed several behavioral abnormalities. When control mice (single transgenics) were suspended by their tails, they spread their hind limbs to prepare for landing. Double transgenic mice, however, showed clasping behavior by pulling their extremities to their body, and this was observed beginning at ~2–3 months of age (Fig. 7A).

In a pen test, control animals that were lowered onto a horizontal pen grabbed it and walked on it, whereas double transgenics were unable to perform this task observed from the age of 6 months onwards (Fig. 7B). Some mice were still able to grab the pen but could not walk on it or could walk on it for only very short time. In fact, more severely affected mice were unable even to sit on the pen; they simply rolled around it because of their strong clasping behavior (Fig. 7B). In addition, some phenotypic double transgenic mice displayed spastic head movements (Fig. 7C). The analysis of footprint patterns of double transgenic mice heterozygous for both the PrP promoter and the SCA3 responder construct (hetero/hetero) showed a normal gait, as did single transgenic controls at the age of 20 months (Fig. 7D). In contrast, age-matched double transgenic mice heterozygous for the promoter and homozygous for the responder (hetero/homo) showed a severely altered gait with a much smaller distance between steps than in controls. When tested at 8 months of age, however, these mice did not yet show this phenotype (Fig. 7D).

To investigate the spontaneous locomotor activity of mice, open-field tests were performed at the age of 6 months. Double transgenic mice that were at least homozygous for the responder construct or for both transgenes were more active than controls or heterozygous mice. Homozygous animals (hetero/homo and homo/homo) moved slightly longer distances (Fig. 8A). Dividing the arena into different subregions, no significant difference between the various genotypes and the controls was observed in the distance covered at the rim of the arena (region 1) (Fig. 8B). The analysis of the inner regions, however, revealed that homozygous mice (hetero/homo and homo/homo) covered significantly longer distances in this area, whereas single transgenic controls as well as heterozygous double transgenics (hetero/hetero) avoided these inner regions. These results are also clearly visible in the exemplary tracking images (Fig. 8C). Only those double transgenic animals that were at least homozygous for the responder or for both transgenes showed higher activity, especially in the inner regions of the arena (Fig. 8D). This may indicate both hyperactivity and reduced anxiety in these mice. In contrast, homozygous mice showed the same activity level as age-matched single transgenic controls.

Although homozygous double transgenic mice showed numerous neurological symptoms, which occurred consistently throughout our analysis, no premature death was observed (data not shown). We next sought to determine the motor activity effects of the ataxin-3 transgene expression. We therefore performed accelerating Rotarod tests on male mice, which allowed us to exclude effects due to the hormone cycle of female mice. Even as early as 9 weeks of age, double transgenic mice showed significant deficits in the accelerating Rotarod test: they could not stay as long on the rotating rod as single transgenic sibling controls (Fig. 9A). Since Rotarod results can also serve as a paradigm for the learning of new movements (motor skill learning) (24), we analyzed the three trials of the first test separately. Although controls as well as double transgenic mice increased the time spent on the Rotarod in consecutive trials, double transgenic mice showed a significantly weaker performance in the third trial. For the control mice, an increase of 34.9 s (±4.3 s) over all three trials was measured, whereas the double transgenic mice improved by only 22.5 s (±5.5 s), indicating that PrP/MJD77 mice display deficits in acquiring new motor strategies (Fig. 9B).

Reversibility of motor symptoms in double transgenic SCA3 mice

Having induced a neurological phenotype in the double transgenic mice, we were interested in whether turning off the ataxin-3 transgene expression would ameliorate the symptoms in phenotypic mice. We therefore divided double transgenic as well as single transgenic mice into two subgroups and treated one group of each genotype with dox starting at the age of 9 weeks.
after calbindin staining. In comparison with single transgenic control mice, double transgenic mice (homozygous for both transgenes—homo/homo) show a tendency towards smaller sizes of Purkinje cells as well as towards reduced molecular layer thickness. However, in age-matched homo/homo mice treated with doxycycline from the age of 2 months onwards, the Purkinje cell sizes and molecular layer thickness was comparable to that measured in control mice.

measure the effect of the block of human ataxin-3 transgene expression. In the first tests, no obvious difference was seen in the performance of untreated versus dox-treated animals. As previously observed, double transgenic PrP/MJD77 mice did not remain as long on the rotating rod as did controls, regardless of whether they were treated with dox or not. However, after approximately 5 months (Rotarod test in the age of 30 weeks, Fig. 9C), the mice treated with dox showed a significant improvement. In this and all subsequent tests, their Rotarod performance was similar to that of single transgenic control mice (Fig. 9C). In contrast, for double transgenic PrP/MJD77 mice without dox treatment, decreasing abilities to remain on the rotating rod were observed with each test (Fig. 9C). Thus, the disease phenotype appeared to worsen in these mice. These findings indicate that turning off the ataxin-3 transgene expression at an early time point indeed leads to an improvement of motor abilities. In fact, since dox-treated double transgenic PrP/MJD77 animals performed as well as control mice after 5 months, our results demonstrate a complete reversal of the Rotarod phenotype in the treated mice.

The analyzed groups of mice contained different genotypes: mice were either heterozygous for both the PrP promoter and the SCA3 responder transgene (hetero/hetero), homozygous for the responder (hetero/homo) or homozygous for both transgene constructs (homo/homo). Analysis of the Rotarod results by genotype showed that the severity of the phenotype depended on the gene dose. For hetero/hetero mice (without dox), no disease phenotype was observed for the duration of the study (1 year). These mice performed similarly as control mice on the Rotarod (data not shown). The results indicating a progressive motor phenotype are therefore based on those animals that are at least homozygous for the responder (hetero/homo) or for both transgenes (homo/homo), respectively. Analysis of the Rotarod performances of dox-treated mice from both of these groups showed a clear improvement for both subgroups, showing that the observed positive effect is independent of the zygosity (Fig. 9D). Taken together, the Rotarod results indicate that a disease phenotype manifests only when a certain gene dose is reached. A motor phenotype was observed only for double transgenic mice that were at least homozygous for the responder (hetero/homo) or homozygous (homo/homo) for both transgenes. In both genotype groups, however, the motor deficit could be reversed by turning off the expression of the ataxin-3 transgene at an early time point.

We also asked whether dox treatment caused any other phenotypic changes in addition to Rotarod performance. Results concerning the body weight of the mice mirrored the results of the Rotarod test; no differences were seen between hetero/hetero PrP/MJD77 mice and single transgenic controls (Fig. 9E), whereas double transgenic mice heterozygous for the promoter and homozygous for the responder (hetero/homo), had significantly lower body weights than controls from the age of 16 weeks onwards (Fig. 9E). Homo/homo mice not only had significantly lower body weights than controls and the hetero/hetero mice, but they were also lighter than age-matched hetero/homo siblings (Fig. 9E). These results indicate that the weaker performances by double transgenic PrP/MJD77 mice in the Rotarod test were not caused by greater body weight. On the contrary, the severity of the phenotype was linked to reduced gain of body weight.

Figure 6. Analyses of cerebellar sections. (A) Electron microscopic analyses of cerebellar Purkinje cells. (I) Single transgenic control mouse (20 months old), normal Purkinje cell with electron-light nucleus and cytoplasm (arrowhead). (II) Double transgenic heterozygous mouse (hetero/hetero) revealing no difference to the control. (III and IV) Double transgenic mice homozygous for one of the transgenes (III, homo/hetero; IV, hetero/homo) showing dark degeneration of Purkinje cells with shrunken, electron-dense cytoplasm and nucleus (arrowheads). In total, nine age-matched mice were analyzed. Darkly stained Purkinje cells were observed in all three transgenic mice analyzed (at least homozygous for one of two transgenes) but in only one of three control mice. In this mouse, only one section of five contained dark stained cells. In two mice, only heterozygous for both transgenes, and one doxycycline treated hetero/homo mouse, no dark cells were detected either. Scale bar, 5 μm. (B) Determination of Purkinje cell size and molecular layer thickness in 21-month-old mice after calbindin staining. In comparison with single transgenic control mice, double transgenic mice (homozygous for both transgenes—homo/homo) show a tendency towards smaller sizes of Purkinje cells as well as towards reduced molecular layer thickness. However, in age-matched homo/homo mice treated with doxycycline from the age of 2 months onwards, the Purkinje cell sizes and molecular layer thickness was comparable to that measured in control mice. Brain sections of three individual mice are included in each group, resulting in a total Purkinje cell number of 880–1250.
Similar to the Rotarod performance, this reduction in body weight was compensated by turning off the expression of the \textit{ataxin-3} transgene. PrP/MJD77 double transgenic mice that were treated with dox at the age of 9 weeks gained as much weight as controls and weighed significantly more than untreated double transgenic mice (Fig. 9F). When the controls were given dox, their body weight remained similar to that of controls that did not receive dox (Fig. 9F). This suggests that the greater gain of body weight of the double transgenic PrP/MJD77 mice (hetero/homo and homo/homo) reflects only the block of \textit{ataxin-3} transgene expression and is not caused by the dox solution, which contains 0.2% sucrose.

\section*{DISCUSSION}

Here we describe the first conditional mouse model of SCA3 generated by overexpressing human \textit{ataxin-3} with 77 CAG repeats under the control of the prion protein promoter. This promoter drives the expression of a responder construct in numerous brain regions such as the \textit{bulbus olfactorius}, the cerebral cortex, the basal ganglia, the \textit{hippocampus} (CA1), the cerebellum and several nuclei of the brain stem (17). In this way, the \textit{PrP} promoter causes \textit{ataxin-3} expression primarily in brain regions known to be altered in SCA3 (reviewed in 3). This model was generated to address the question of whether a neurological phenotype once present in the mice would be reversible after turning off the transgene expression. Such reversal has been shown in similar inducible mouse models of HD and SCA1 (23,25). However, polyglutamine diseases differ both in their clinical manifestations and in their pathogenesis (26,27). Moreover, in an inducible mouse model of Parkinson’s disease overexpressing alpha-synuclein, we were not able to reverse the phenotype, although we could stop disease progression (18).

In the present study, we found that one responder line, 2904, expressed \textit{ataxin-3} in many regions throughout the entire brain with the strongest transgene signals in the cerebellum. On the cellular basis, \textit{ataxin-3} was predominantly detected in glial cells of the cerebellum. Halting the production of the pathogenic \textit{ataxin-3} protein using dox treatment for 2 weeks led to the complete absence of the transgenic protein without any residual expression, indicating that this line is ideal for studying the reversibility of a phenotype.

The conditional SCA3 model reflects many features of the human condition. First, in neuronal cells of the cerebral cortex, intranuclear inclusions staining with antibodies directed against \textit{ataxin-3} were detected in a gene dose-dependent manner. Intranuclear inclusion bodies are a hallmark of many polyglutamine diseases including SCA3 (3,6,7). Nevertheless, in the case of the strongly expressing glial cells of the cerebellum, their small cell bodies made it difficult to determine whether they also contained inclusions. However, these cells strongly overexpress human transgenic \textit{ataxin-3}.

Although Purkinje cells do not express the pathogenic \textit{ataxin-3} transgene, an altered function of Purkinje cells was detected in double transgenic PrP/MJD77 mice. The role of Purkinje cell degeneration in SCA3 remains controversial. Studies on patients have revealed a reduction in the number of Purkinje cells. On the other hand, the number of granule cells in the cerebellum of double transgenic mice was not significantly reduced, suggesting that the role of the cerebellum in SCA3 is not as pronounced as in the case of HD or SCA1.

\begin{figure}[h]
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\caption{Behavioral symptoms of PrP/MJD77 mice. (A) A double transgenic PrP/MJD77 mouse showing clasping behavior when suspended by the tail, whereas a single transgenic mouse (control) displays the normal behavior of spreading the hind limbs to prepare for landing. (B) When performing a pen test, control mice are able to grab a pen and walk on it. Phenotypic PrP/MJD77 mice, however, cannot grab and walk on the pen and therefore fail to perform this test. (C) In some double transgenic PrP/MJD77 mice, spastic movements of the head can be observed. (D) Analysis of the footprint pattern of double transgenic PrP/MJD77 mice heterozygous for both transgenes (hetero/hetero) revealed no obvious changes compared with an age-matched single transgenic control at the age of 20 months. In addition, 8-month-old PrP/MJD77 mice heterozygous for the promoter, but homozygous for the SCA3 responder (hetero/homo) showed a normal footprint pattern. However, when this test was repeated in the age of 20 months, clear disturbances in the gait became visible. When compared with age-matched controls, the footprint patterns of these mice were uncoordinated and the distances between the steps were strongly reduced.}
\end{figure}
of Purkinje cells (28–30), and degeneration has been observed in several mouse models of SCA3 (8,10,12). Other studies, however, have not confirmed a noticeable loss of these cells in SCA3 patients (31,32).

The frequently used mouse prion protein promoter construct (33) shows no activity in Purkinje cells, as established in many mouse models (8,33,34). Nevertheless, the degeneration of Purkinje cells has been observed in transgenic mouse models of SCA7 and SCA3 that use this promoter (8,34). It is possible that in our PrP/MJD77 model, as well as in the previously mentioned models, the absence of neurotrophic factors from damaged cells, at the synapses of Purkinje cells, leads to the observed degeneration of these cells (34).

The second main feature resembling the human disease is the comprehensive behavioral phenotype in the mice. We observed claspng behavior, gait disturbances and spasticity. Disturbances in the coordination of movements became obvious, e.g. in a pen test with 6 months of age. Several double transgenic PrP/MJD77 mice presented with claspng behavior already at 2–3 months of age. More severe motor deficits as revealed by accelerating Rotarod analyses became obvious at the age of 9 weeks in double transgenic PrP/MJD77 mice that were at least homozygous for the responder construct (n = 12) moved longer distances than age-matched single transgenic controls (n = 20) and also than heterozygous mice (n = 6). (B) No significant differences were seen in the distances that all three groups moved at the outer rim of the arena (region 1). (C) Representative movement tracking images of a single transgenic control and PrP/MJD77 mice with different genotypes. There were no differences between the control and the hetero/hetero mouse. The hetero/homo and the homo/homo mouse, on the other hand, showed increased spontaneous locomotor activity especially in the center of the arena. (D) In region 2, the transition region between the edge (region 1) and the center (region 3) of the arena, hetero/homo and homo/homo transgenic mice covered signficantly longer distances than controls and hetero/hetero mice (**P < 0.05). For double transgenic PrP/MJD77 mice, a higher movement activity was observed, mainly due to the distance they moved in the center of the arena. The behavior of hetero/hetero double transgenic mice, however, did not vary from that observed for control animals. Shown here are results from male mice. Comparable results were obtained for female mice. Error bars, \( \pm \) SEM.

![Open field test at the age of 6 months. (A) The total distance mice moved through the arena in the first 8 of 15 intervals (1 interval = 1 min). Double transgenic PrP/MJD77 mice that were at least homozygous for the responder construct (n = 12) moved longer distances than age-matched single transgenic controls (n = 20) and also than heterozygous mice (n = 6). (B) No significant differences were seen in the distances that all three groups moved at the outer rim of the arena (region 1). (C) Representative movement tracking images of a single transgenic control and PrP/MJD77 mice with different genotypes. There were no differences between the control and the hetero/hetero mouse. The hetero/homo and the homo/homo mouse, on the other hand, showed increased spontaneous locomotor activity especially in the center of the arena. (D) In region 2, the transition region between the edge (region 1) and the center (region 3) of the arena, hetero/homo and homo/homo transgenic mice covered significantly longer distances than controls and hetero/hetero mice (**P < 0.05). For double transgenic PrP/MJD77 mice, a higher movement activity was observed, mainly due to the distance they moved in the center of the arena. The behavior of hetero/hetero double transgenic mice, however, did not vary from that observed for control animals. Shown here are results from male mice. Comparable results were obtained for female mice. Error bars, \( \pm \) SEM.](http://example.com/image.png)
Figure 9. Rotarod analyses and body weight of PrP/MJD77 mice. (A) Rotarod test of PrP/MJD77 double transgenic mice at the age of 9 weeks. Single transgenic controls (transgenic only for the responder construct, white column, n = 22) were able to stay significantly longer (***P < 0.005) on the rotating rod than double transgenic mice (gray column, n = 19). (B) To evaluate the ability to learn new movement patterns (motor skill learning), we analyzed the results of the three trials of the Rotarod test at the age of 9 weeks separately. Controls as well as double transgenic mice (PrP/MJD77) increased the time spent on the Rotarod over the three trials. However, in the third trial, the double transgenic mice did not improve as much as the controls (**P < 0.05). Over all three trials, the controls improved their performance by 34.9 s (± 4.3 s) but the PrP/MJD77 mice only by 22.5 s (± 5.5 s). (C) Results of the Rotarod test conducted approximately every 6 weeks for 1 year to measure the development of the motor phenotype in untreated double transgenic mice (PrP/MJD77, n = 9) compared with controls (controls, n = 11), as well as doxycycline-treated double transgenics (PrP/MJD77-dox, n = 10) and treated controls (controls-dox, n = 11). Doxycycline treatment started at the age of 9 weeks. After a period of ~5 months of treatment (at an age of ~30 weeks), dox-treated double transgenic mice showed significant improvement in their Rotarod performance compared with the untreated double transgenics which displayed a progressive motor phenotype (***P < 0.005, ***P < 0.0005). (D) Double transgenic mice that were hetero/homo or homo/homo (n = 5) showed a progressive decline in their Rotarod performance. Doxycycline-treated mice of these genotypes (n = 6), however, showed a significant improvement in their Rotarod performances after 5 months of doxycycline treatment (***P < 0.005). (E) Analysis of the body weight of the mice tested on the Rotarod revealed that hetero/hetero mice (n = 4) did not show differences in their weight compared with age-matched single transgenic controls (n = 11) over 1 year. Hetero/homo double transgenic mice (n = 2), on the other hand, weighed significantly less, with homo/homo mice (n = 3) showing the lowest gain of body weight. In fact, these mice even weighed significantly less than hetero/homo mice (**P < 0.005). (F) Doxycycline-treated hetero/homo and homo/homo mice (n = 6) gained significantly more weight than untreated mice of the same genotype groups (n = 5); in fact they gained as much weight as the control mice (**P < 0.005). Error bars, ± SEM.
SCA3, since the first signs of altered motor coordination became obvious at the age of 2 months, whereas degeneration of Purkinje cells was observed at the age of 20 months. In various transgenic mouse models of SCA1, in which cell death affects primarily Purkinje cells, ataxic symptoms manifest before a significant loss of Purkinje cells is observed (23,37,38). Similar results have been shown in other mouse models of SCA3 (8,11,12).

In addition to the frontoparietal cortex and the basal ganglia, the cerebellum also seems to play an important role in the learning of new motor skills (39). In young mice of the PrP/MJD77 model, mild deficits in motor skill learning were observed. The slightly impaired learning of new movements may therefore result from ataxin-3 transgene-related defects in the circuit of cortex and cerebellum. A dysfunction of cerebrotectical neurons in SCA3 patients has been proposed previously (40). In contrast to other published models of polyglutamine diseases, mutant ataxin-3 is expressed in glial cells in the cerebellum in our model. In this way, our model provides evidence that the expression of an expanded polyglutamine protein in glial cells alters function of Purkinje cells. This phenomenon has already been described for other neurotoxic proteins such as alpha-synuclein that, when expressed in mine protein in glial cells alters function of Purkinje cells. In this way, our model proposes a mechanism for how the ataxin-3 transgene-related defects observed. The slightly impaired learning of new movements may therefore result from ataxin-3 transgene-related defects in the circuit of cortex and cerebellum. A dysfunction of cerebrotectical neurons in SCA3 patients has been proposed previously (40). In contrast to other published models of polyglutamine diseases, mutant ataxin-3 is expressed in glial cells in the cerebellum in our model. In this way, our model provides evidence that the expression of an expanded polyglutamine protein in glial cells alters function of Purkinje cells.

Having established a comprehensive phenotype in PrP/MJD77 mice, we investigated whether the symptoms can be reversed by turning off transgene expression. If the disease phenotype in mice turns out to be irreversible even after turning off the toxic agent completely, it may not prove possible to find a cure for severely affected patients. In this respect, our data are highly encouraging also for patients. Although accelerated Rotarod tests showed no improvement for the treated mice during the first 4 months, long-term treatment for at least 5 months led to a significant improvement, which persisted until the latest time point in the study (1 year). Most importantly, the motor abilities of the treated mice were similar to those of single transgenic controls. On the other hand, for untreated phenotypic mice (hetero/homo and homo/homo), progressive deterioration of the motor phenotype was noticeable. Thus, our results indicate that 5 months of turning off the expression of the pathogenic ataxin-3 transgene reverses the motor coordination deficit in phenotypic PrP/MJD77 mice. In addition to rescuing motor coordination ability, long-term block of ataxin-3 expression restored the body weight to control levels. Double transgenic PrP/MJD77 mice of the genotypes hetero/homo and homo/homo, gained significantly less weight compared to hetero/hetero mice and single transgenic controls. Over time, however, dox-treated hetero/hetero and homo/homo mice gained weight and ended up with body weights similar to those of control animals. The reduced gain of weight most likely correlates with the hyperactivity that we measured in our open field test and that has also been reported for conventional SCA3 mouse models (12).

Our data indicate that preventing the expression of the expanded ataxin-3, such as by using allele-specific RNA interference demonstrated recently in a lentiviral-generated rat model (14), may be an approach to treat SCA3 patients. As promising as these data are, our findings and the results obtained for other inactivatable polyglutamine diseases such as HD and SCA1 (23,25) clearly indicate that treatment at early disease stages may have clear-cut benefits. In this way, our study has provided valuable information for designing clinical trials in human patients.

MATERIALS AND METHODS

Transgenic mice

To generate a transgenic mouse model using the Tet-Off system (15), two constructs are necessary: the promoter construct, which controls the expression of a tTA, and a responder construct, which harbors the gene-of-interest under the control of a minimal promoter. In double transgenic mice, the tTA binds to the tetracycline-responsive element (TRE) in the responder construct and thereby enables the expression of the gene-of-interest. Tetracycline or one of its derivatives can bind to the tTA and inhibit the binding of the transactivator to the TRE, thereby blocking the expression of the gene-of-interest (15) (Fig. 1). In order to generate double transgenic mice, we used the hamster prion protein promoter mouse line for the expression of tTA [Tg(Prnp-tTA)FVByPrnp0/0], which was kindly provided by S. Prusiner (Institute for Neurodegenerative Diseases, San Francisco, USA) (16). These mice are referred to as PrP in this study.

SCA3 responder mice were generated using a XhoI/BstEII fragment of the pUHD10-3 expression vector (43), which contains full-length ataxin-3 cDNA (ataxin-3c isoform) (19) with 77 CAG repeats downstream of the tTA-responsive promoter (containing a TRE). The responder DNA fragment was injected into fertilized oocytes using standard procedures. Mouse lines were established by crossingbreeding with C57BL/6 mice. Transgenic animals were identified using DNA extracted from ear or tail biopsy tissue (Roche High Pure PCR Template Preparation Kit, Roche Applied Science, Mannheim, Germany). PCR analyses were carried out for promoter mice using primers specific for the TTA sequence: tTA-F, 5'-GAC GAG CTC CAC TTA GAC GG-3'; tTA-R, 5'-TAC TCG TCA ATTCCA AGG GC-3'. PCR analyses of responder mice were carried out with primers specific for the TRE vector: TRE-Seq/PCR-F, 5'-CGC CTG GAG AGC CCA TCC-3'; TRE-Seq/PCR-R, 5'-CCA CAC CTC CCC CTG AAC-3'.

Mice were kept on a 12 h light/dark cycle and had ad libitum access to food and water. To block ataxin-3 transgene expression, mice were treated with 2 mg/ml dox (Sigma-Aldrich, Munich, Germany) in drinking water supplemented with 0.2% sucrose. To evaluate the consequences of long-term sugar-enriched dox treatment, the blood sugar level was checked (A. Menarini Diagnostics, Wien, Austria). The blood glucose level of mice treated for up to 11 months did not vary from that measured in age-matched untreated animals (data not shown).

Fragment analysis

To determine the number of CAG repeats in the ataxin-3 transgene, fragment analysis was performed. In a PCR reaction, a fragment containing the CAG repeats was amplified using the following primers: hMJD1c-CAG-Frag-F, 5'-Cy5-GCT
AAG TAT GCA AGG TAG TTC C-3'; hMJD1c-postGAG-R, 5'-CAA GTG CTC CTG AAC TGG TG-3'. The indodicarbocyanine (Cy5) labeled PCR products were supplemented with an internal standard (DNA-Size Standard Kit-600, Beckman Coulter, Krefeld, Germany) and separated and analyzed on the CEQ8000 Genetic Analysis System (Beckman Coulter). Plasmid DNA of ataxin-3 cDNA containing 15, 77 or 148 CAG repeats was used as a size standard to calculate the number of CAG repeats.

Western blot
Mice were sacrificed by CO₂ inhalation, and the tissue was freshly prepared, immediately snap frozen, and stored at −80°C. For protein isolation, tissue was homogenized at 30 000 r.p.m. using a tissue homogenizer (Ultra-Turrax; IKA Werke, Staufen, Germany) in TES buffer (50 mM Tris, pH 7.5, 2 mM EDTA and 100 mM NaCl) supplemented with a protease inhibitor cocktail (Complete, Roche). After the addition of Nonidet P-40 (Sigma-Aldrich) to a final concentration of 1% and incubation at 4°C for 15 min, debris was removed by centrifugation (15 min each, 20 000 relative centrifugal force, 4°C). The clarified protein extract was supplemented with glycerol (VWR International, Darmstadt, Germany) to a final concentration of 10% and stored at −80°C. For the determination of the protein concentration, a protein assay (Protein Assay Dye Reagent Concentrate; Bio-Rad, Munich, Germany) based on the method described by Bradford (44) was used according to the manufacturer’s instructions. Protein lysates (30 μg of each) were mixed with loading buffer (80 mM Tris pH 6.8, 0.1 mM DTT, 2% SDS, 10% glycerol, bromophenol blue), denatured and analyzed in PAGE buffer (192 mM glycine, 25 mM Tris, 1% SDS) by SDS–PAGE (Blue Vertical 100/C; Serva, Heidelberg, Germany) according to the method described by Laemmli (45). Separated proteins were transferred to polyvinylidenedifluoride membranes (Immobilon-P Transfer membrane, Millipore, Schwabach, Germany) in transfer buffer (0.2 mM glycine, 25 mM Tris, 10% methanol). The detection of proteins was performed essentially as described previously (7). Briefly, the membrane was blocked for 2 h at room temperature in 5% dry milk in TBST buffer (10 mM Tris, pH 7.5, 0.15 M NaCl, 0.1% Tween-20). The primary antibody was diluted in TBST. The generation of our anti-ataxin-3 antibody (diluted 1:1000) has been described previously (7). The 1H9 antibody against ataxin-3 and the β-actin antibody were purchased from Chemicon (Hofheim, Germany) and Sigma-Aldrich, respectively. After incubation for 2 h, the membrane was washed four times (15 min each) with TBST. The membrane was incubated for 75 min with a secondary antibody that was coupled to horseradish peroxidase (GE Healthcare, Freiburg, Germany). After four washing steps with TBST (15 min each), bands were visualized using the enhanced chemiluminescence method (ECL; GE Healthcare) and exposure to Hyperfilm ECL (GE Healthcare).

Immunohistochemistry and immunofluorescence
Mice were deeply anesthetized by CO₂ inhalation and transcardially perfused using 4% paraformaldehyde in 0.1 M PBS. Brains were removed from the skull and post-fixed overnight in fixative, embedded in paraffin and cut into 7 μm sagittal sections. Immunohistochemical staining of paraffin-embedded tissue was performed as described previously (46). Briefly, after rehydrating the sections in xylene and a graded alcohol series, slides were, if necessary, microwaved for 15 min in 10 mM sodium citrate, pH 6.0. Slides were washed with PBS, and endogenous peroxidases were blocked using 1% hydrogen peroxide in 40% methanol for 10 min, followed by 5% normal goat serum in PBS supplemented with 0.3% Triton X-100. After washing with PBS three times for 10 min each, the primary antibody (diluted in PBS containing 3% goat serum) was added and incubated at 4°C overnight in a humid chamber. After washing the slides with PBS, the secondary antibody coupled with biotin (Vector Laboratories, Burlingame, CA, USA) and diluted in PBS containing 1.5% goat serum was added and incubated for 30 min at room temperature. After a brief wash with PBS, sections were incubated in an ABC enhancer complex coupled with peroxidase (Vector Laboratories) for 30 min at room temperature. After washing with PBS, the substrate (DAB; Sigma-Aldrich) was added, and the reaction was stopped in distilled water once the desired degree of staining was reached. Finally, slides were dehydrated again and mounted using CV Mount (Leica, Bensheim, Germany). The antibody against calbindin (CB-38a) was purchased from Swant (Bellinzona, Switzerland).

Staining was visualized using an Axiosplan 2 imaging microscope (Carl Zeiss Microimaging, Oberkoehmen, Germany) equipped with an Axio-Cam MR color digital camera (Carl Zeiss Microimaging) using a 40× Plan Neofluar and a 63× Plan/Apochromat objective and the AxioVision 4.6 software package (Carl Zeiss Microimaging).

For statistical analysis of the size of Purkinje cells and the thickness of the cerebellar molecular layer, calbindin stained brain sections of three individual mice were included in each analyzed group, resulting in a total Purkinje cell number of 880–1250.

The double immunofluorescence staining of paraffin-embedded sections was performed essentially as described above, but with normal donkey serum used for blocking. The NeuN antibody against neuronal cells was purchased from Chemicon and the antibody against GFAP was purchased from DAKO (Hamburg, Germany). Secondary antibodies were Cy2-coupled anti-rabbit and Cy3-coupled anti-mouse (Dianova, Hamburg, Germany). After incubation with the secondary antibodies and washing with PBS, sections were coverslipped with Mowiol (Merck, Darmstadt, Germany) supplemented with 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma-Aldrich) and stored at 4°C. To visualize staining, a Zeiss LSM 510 microscope (Carl Zeiss Microimaging) was used with suitable filter sets.

Electron microscopy
Electron microscopic analyses were performed essentially as described previously (47). After being deeply anesthetized by CO₂ inhalation, mice were transcardially perfused using 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Brains were removed and post-fixed in fixative for 30 min to 1 h. After dehydration in ethanol, brain pieces were incubated overnight in 70% ethanol saturated with uranyl acetate, followed by an additional dehydration in absolute ethanol and propylene
oxide. Finally, the samples were embedded in Araldite 502 (Sigma-Aldrich) and sectioned on a Leica FCR Ultracut ultramicrotome (Leica). Ultrathin sections were stained with lead citrate and examined with a Zeiss EM 10 electron microscope. At least five sections per sample were analyzed.

Open field analysis
For assessment of explorative behavior and emotionality, open field tests were performed. Mice were placed in a 50 × 50 cm arena with 50 cm high walls and their movement activity was recorded for 15 min using the TSE VideoMot2-Video Activity Tracking System (TSE Systems, Bad Homburg, Germany). The light intensity was set to be at least 150 lux in the corners and not more than 200 lux in the center of the arena (48,49). To analyze the collected data, the arena was divided into different regions. Region 1 is the rim with a width of 8 cm, region 2 the center that comprises 16% of the overall area and region 3 the area between the rim and the center.

Rotarod
To measure the motor coordination abilities and balance of the transgenic mice, Rotarod analyses were performed. At a maximum illumination of 100 lux, mice were tested on an accelerating Rotarod (TSE Systems, Bad Homburg, Germany), which began at 4 r.p.m. and accelerated to 40 r.p.m. over 300 s (5 min). Three trials per test day were carried out, with a 15 min rest between trials. In each trial, the latency to fall off the rotating rod was recorded (48,49). The tests were repeated approximately every 6 weeks.

Footprint analysis
The gait of the mice was analyzed using footprint patterns. The fore paws of the mice were stained with red and the hind paws with blue non-toxic paint. Mice were placed on a sheet of paper (A3 size) in front of a tunnel (5 × 5 × 60 cm). Middle steps of the track were compared for the distance between steps.

Pen test
In the pen test, a mouse suspended by its tail was slowly lowered from above to a pen (diameter, ~1 cm) horizontally fixed ~25 cm above the ground. Usually, the mouse grabs for the pen and starts walking on it without difficulties. Any deviation from this normal behavior was recorded.

Statistical analysis
Data are presented as mean ± SEM. Results of cerebellar measurements after calbindin immunohistochemistry, open field tests and Rotarod tests were analyzed using Student’s t-test with a significance threshold of P < 0.05.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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