

CHAPTER 5

RESULTS

5.1. Previous study: cell culture and organotypical slices

Initial experiments have been conducted to ensure that the tet-on system works. A neuronal cell culture from mice expressing rtTA was transfected with a construct containing tet promoter, CGG and GFP reporter. These cells showed expression of GFP after dox treatment (data not shown). It was concluded that this system worked *in vitro* and was ready to make the transgenic mice and cross them producing bigenic mice.

From the bigenic mice produced, this system was verified whether it also worked in the bigenic mice organs, especially the brains where the expression was expected. Organotypical cultures were conducted to prove it. The brains of bigenic mice Tet-on-99CGG-eGFP/GFA2-rtTA and Tet-on-99CGG-eGFP/PrP-rtTA at 6 days old were taken. Brain slices were cultured and treated with dox, and expression of GFP was observed under (confocal) microscope. The cultures showed strong GFP expression throughout the brain (data not shown). This result gives an insight that those bigenic systems work *in vivo*.

5.2. The workable of Tet-on-nCGG-eGFP: *in vivo* study using Tet-on-nCGG-eGFP/hnRNP-rtTA

In vivo experiment then was started. As a proof of principle, bigenic mice Tet-on-nCGG-eGFP/hnRNP-rtTA were used and treated with dox in drinking

water. hnRNP-rtTA was used since these mice have previously been used and showed ubiquitous expression in all tissues (62). The bigenic mice with Tet-on-99CGG-eGFP/hnRNP-rtTA exhibited weight loss during 4 days of dox treatment, and died after 5 days of dox in drinking water treatment, while mice with Tet-on-11CGG-eGFP/hnRNP-rtTA and monogenic controls were normal, confirming expression of 99CGG as the cause of the death, neither the GFP nor the dox treatment.

Immunohistochemistry was performed to investigate the expression of GFP reporter of the transgene in several different tissues. The analysis showed GFP expression in liver, kidney, heart, lung, intestine, stomach, spleen, brain, testis of the bigenic mice after 4 days dox treatment (Figure 3). GFP expression in the bigenic mice Tet-on-nCGG-eGFP/hnRNP-rtTA without dox treatment as well as in monogenic mice Tet-on-99CGG-eGFP with/without dox treatment was not found, indicating no leakage of expression occurred in our Tet-on system. Unfortunately using the similar experiment, the transgenic line Tet-on-11CGG-eGFP from the only founder we have, founder 11, exhibited loss of GFP expression after several breedings, hence this line could not be used anymore (data not shown). This experiment has proven that basically the inducible transgenic system worked *in vivo*. It had to be found out whether this inducible transgenic system works *in vivo* using our most important driver promoters, the GFA2 and PrP.

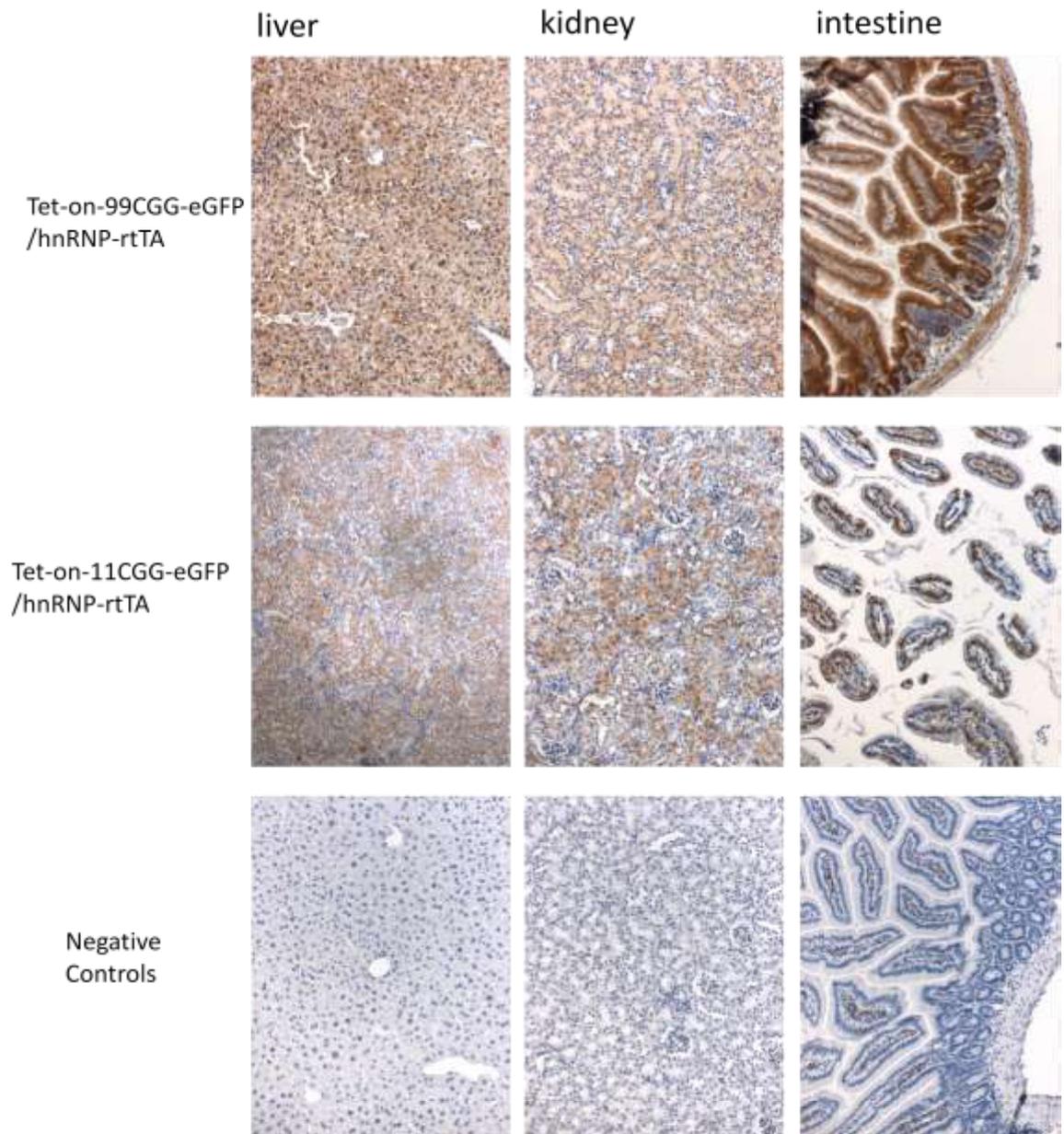


Figure 3. Immunohistochemistry for GFP in liver, kidney, and intestine of bigenic mice Tet-on-nCGG-eGFP/hnRNP-rtTA with dox treatment. Positive results are indicated with the presence of brown color, which means the tissues express the target gene. Bigenic mice Tet-on-nCGG-eGFP/hnRNP-rtTA with dox induction expressed the transgene. While control mice (wild type, monogenic mice, and bigenic mice without dox) did not show any staining for GFP (no brown color), which means these controls did not express the transgene. 200X magnification.

5.3. The workable of Tet-on-99CGG-eGFP/GFA2-rtTA *in vivo*

The transgene expression of bigenic mice Tet-on-99CGG-eGFP/GFA2-rtTA were then tested *in vivo* by giving dox in drinking water. After 6 weeks of dox in drinking water, these bigenic mice were sacrificed and the brains were collected. Immunohistochemistry, Western blot and RT-Q-PCR for GFP were performed to check the expression. The result was disappointing. Immunohistochemistry did not show any signal of GFP in any brain regions. Western blot and RT-Q-PCR did not detect the expression either. To rule out the deficiency of the inducer, the dox treatment was lengthened until 9 weeks, of which a dose of 10 mg/ml of dox during the last 4 days was used, but still there was no expression detected. The rtTA expression in the brain of these bigenic mice was checked by rtTA immunohistochemistry. Apparently the absence of expression was due to the absence of rtTA expression, since the rtTA immunohistochemistry did not show any rtTA expression (data not shown).

5.4. The workable of Tet-on-99CGG-eGFP/PrP-rtTA *in vivo*

The feasibility of another bigenic line, the Tet-on-99CGG-eGFP/PrP-rtTA, was checked *in vivo* by administration of dox in drinking water. After 2 weeks of dox treatment, these mice were sacrificed, and GFP expression in the brains was analyzed by immunohistochemistry. Immunohistochemistry for GFP showed expression in the brain. GFP was expressed in a mosaic pattern, mainly in hippocampus, striatum, and cerebellum (Figure 4). RT-Q-PCR and Western blot

for GFP were performed to the RNA and protein extracted from the brains of these mice. Although GFP was expressed in only a small part of the brain, the GFP expression could still be detected by the RT-Q-PCR as well as the Western blot (Figure 14 and 15 sample A). This finding suggested that the transgene system for this bigenic line worked *in vivo*, and is ready to be used for FXTAS neuropathological studies. Before the main experiment and further studies using these bigenic lines were started, it was necessary to choose the best founders of the PrP-rtTA and the Tet-on-99CGG-eGFP, since there were several founders for both of the transgenic lines. The best founders were expected to be the transgenic lines which give the strongest and appropriate expression, and thus be able to produce enormous expression of expanded premutation CGG transcript. The abundant expanded premutation CGG expression is expected to provide the sufficiency to produce severe form of FXTAS outcomes.

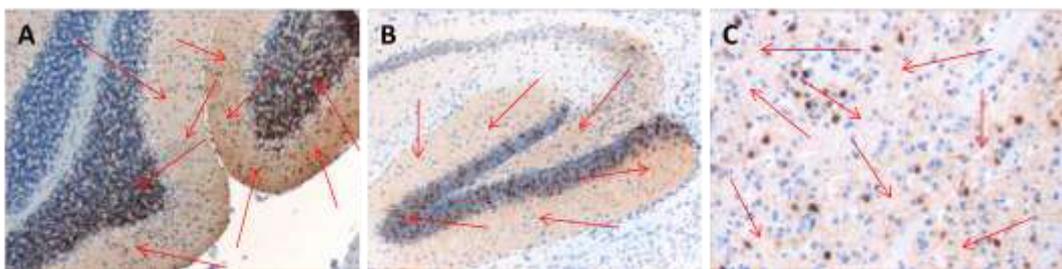


Figure 4. Immunohistochemistry for GFP in brain slices of bigenic mice Tet-on-99CGG-eGFP/PrP-rtTA after 2 weeks of dox in drinking treatment. Positive results are indicated with the brown color staining (red arrow), which means the tissues express the transgene. The bigenic mice Tet-on-99CGG-eGFP/PrP-rtTA expressed the transgene in the brain after dox treatment. The expression in the brain was mainly found in cerebellum (A), hippocampus (B), and striatum (C). 100X magnification.

5.5. Transgene expression outside the brain in Tet-on-99CGG-eGFP/PrP-rtTA bigenic mice

One other important thing for further studies using this bigenic line was to make sure the expression of the transgene was restricted in certain cells in respect of the driver promoter. For the Tet-on-99CGG-eGFP/PrP-rtTA mice, the expression of expanded premutation CGG-eGFP was expected only in all brain cells except for the Purkinje cells. To assess the possibility of leakage of expression in other tissues outside the brain, the fastest way was by treating the mice with dox by intraperitoneal injection. Afterwards the mice were sacrificed, and the tissues were collected to be analyzed for the presence of GFP by immunohistochemistry. The dox dose was 500 microgram, and was given 2 times in 48 hours. Immunohistochemistry revealed the presence of GFP in the kidney, in the proximal tubule to be precise. Other tissues such as heart, intestine, liver, lung, and stomach did not show any GFP staining (Figure 5). To confirm the finding of expression in the kidney, the GFP expression in kidney was checked using Western blot and RT-Q-PCR. Similar to the immunohistochemistry result, Western blot and RT-Q-PCR proved the presence of GFP expressions in the kidney of these bigenic mice (Figure 6 & 7). The leakage in kidney was also observed by RT-Q-PCR and Western blot when these bigenic mice were treated with 2 weeks of dox in drinking water treatment (data not shown).

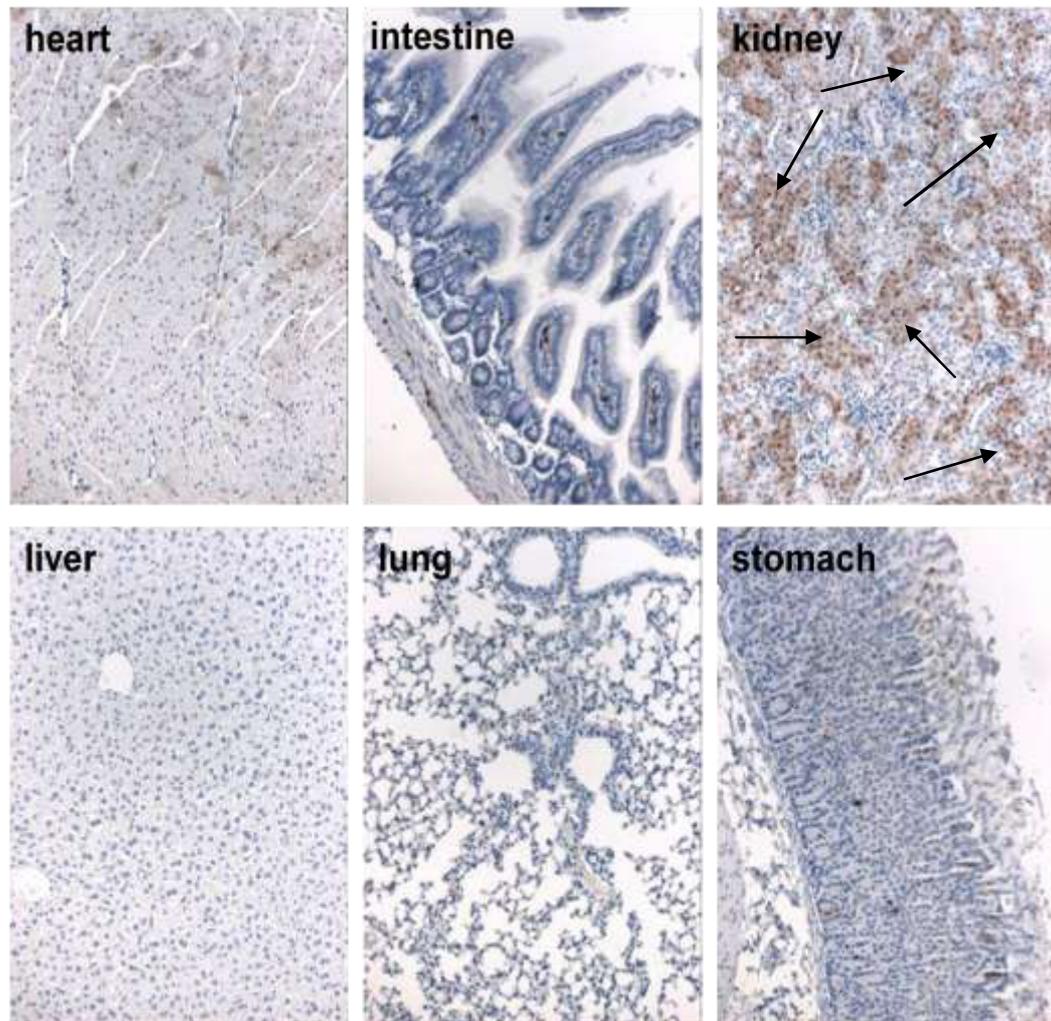


Figure 5. Immunohistochemistry for GFP in tissues outside the brain of bigenic mice Tet-on-99CGG-eGFP/PrP-rtTA after dox intraperitoneal injection. GFP expression is shown with the brown staining (arrow). The leakage was only seen in the kidney, but not in the rest. Other tissues but kidney did not show any brown staining, negative results. 200X magnification.

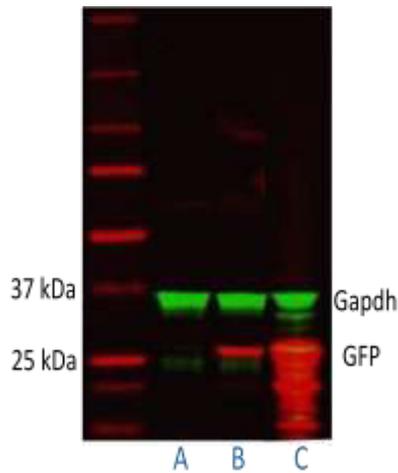


Figure 6. Western blot for GFP on kidney of tet-on-99CGG-eGFP/PrP-rtTA mouse after dox IP treatment. The red bands are GFP; the green bands are Gapdh as the internal control. We could see the kidney of this bigenic mouse (B) expressed the GFP, indicated by the presence of red band. Sample A was the negative control. Sample C was the positive control.

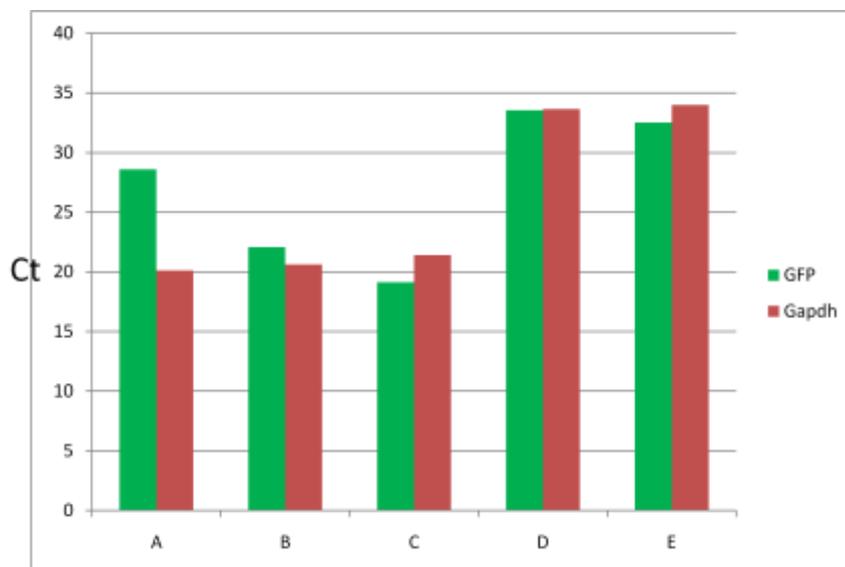


Figure 7. RT-Q-PCR for GFP RNA from kidney of tet-on-99CGG-eGFP/PrP-rtTA mouse after dox IP treatment. Green bars represent the GFP. Red bars were Gapdh which was used as internal control. Kidney of bigenic mice (B) and positive control (C) expressed the GFP transgene, indicated with the low Ct value. High Ct value, as seen in negative controls (A, D, and E), means the samples did not express the GFP transgene. A is the kidney of monogenic mouse, D is a control without reverse transcription and E is the water control.

5.6. Best founder of PrP-rtTA

For the PrP-rtTA transgene, there were two founders available, founder 1 and founder 3. To choose the best founder to be used for further study, the rtTA expressions between these two founders were compared by RT-Q-PCR. RT-Q-PCR for rtTA was assayed to the mRNA isolated from the brain of both founders to compare the rtTA levels. The RT-Q-PCR showed more rtTA transcript in founder 3 than founder 1 (Figure 8). The rtTA mRNA levels of founder 3 were about 4 fold higher than founder 1. Based on this observation founder 3 gave more expression of rtTA than founder 1, and founder 3 of PrP-rtTA line was chosen for further studies instead of founder 1.

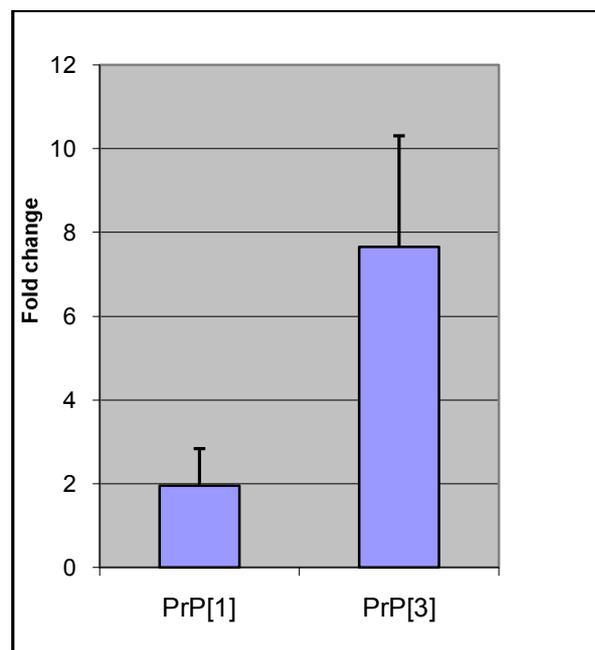


Figure 8. rtTA mRNA levels from the brains of different PrP-rtTA founder. rtTA expression of founder 3 was found to be almost 4 times higher than founder 1. The rtTA expression in founder 3 was stronger than founder 1. In this experiment 4 mice of each founder were used.

5.7. Best founder of Tet-on-99CGG-eGFP

The best founder of Tet-on-99CGG-eGFP needed to be chosen. There were two founders available, founder 31 and founder 39. The strategy to come to the decision was by creating bigenic mice Tet-on-99CGG-eGFP/hnRNP-rtTA derived from both different founders of Tet-on-99CGG-eGFP. The bigenic mice were treated with dox water or food treatment for 4 days to induce the expression of the transgene. GFP expression in liver of the mice from those different founders, were compared by immunohistochemistry, Western blot and RT-Q-PCR. From the immunohistochemistry of the liver, the bigenic mice from founder 39 seemed to give the expression in mosaic pattern, with many cells not expressing the transgene. Expression of the transgene in founder 31 was found more homogenous throughout the cells, and gave stronger signal than founder 39 (Figure 9).

Although the RT-Q-PCR showed variation of RNA levels within the same founder, generally the expression on founder 31 is higher than founder 39. The transgene RNA expression levels of founder 31 were about 4-fold higher in the liver compared to founder 39 (Figure 10). From the Western blot and RT-Q-PCR in the liver, founder 31 gave stronger expression of GFP than founder 39. The Western blot on liver showed much stronger signal in founder 31 than founder 39. The GFP protein levels in the liver of founder 31 were about 12-fold higher than founder 39 (Figure 11). Since it was the same hnRNP-rtTA line for both founders, it was assumed that the difference of expression was due to the difference of Tet-

on-99CGG-eGFP founder. Based on the results, it was decided to use founder 31 for further studies, although it was still possible to use the founder 39.

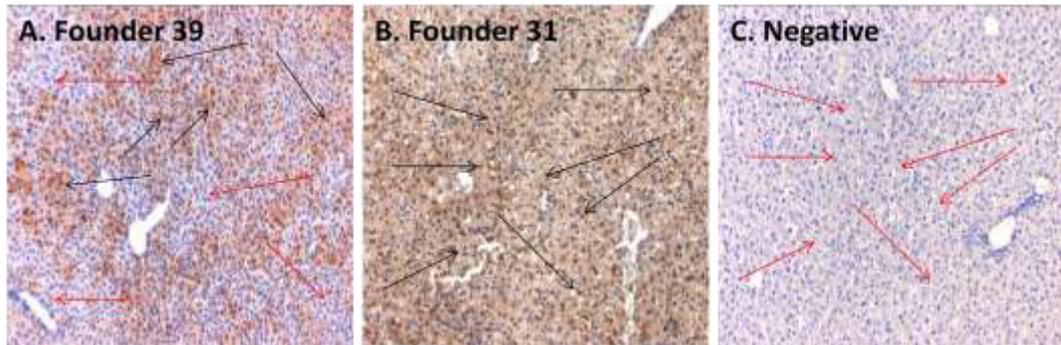


Figure 9. Immunohistochemistry for GFP in the liver slices comparing founder 31 and 39 of Tet-on-99CGG-eGFP. Positive results were indicated with the presence of brown color staining (black arrow), which means the tissues expressed the transgene. The expression in founder 31 (B) was much more homogenous with more cells expressed the GFP than founder 39 (A). In founder 39 (A) there were many cells which did not express the transgene (pointed with red arrow). Sample C is negative control which was negative for GFP staining, which was indicated with the blue color staining. 200X magnification.

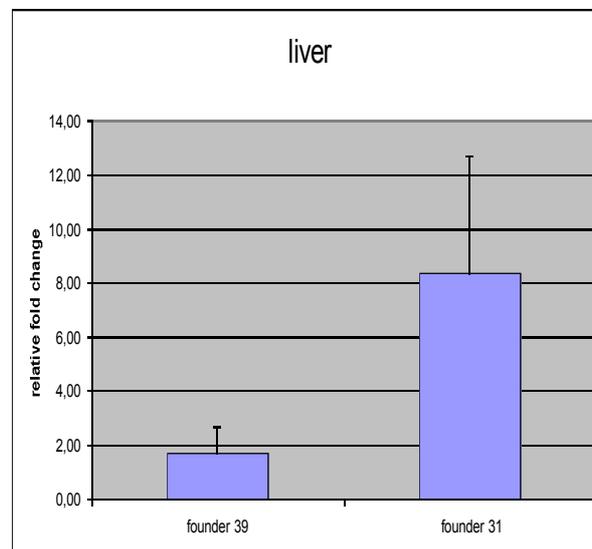


Figure 10. GFP RNA levels in the liver comparing founder 31 and 39 of Tet-on-99CGG-eGFP. Founder 31 gave stronger expression than founder 39. It was more than 4 times higher in founder 31. Founder 31 was considered as the best founder because of the strong expression of transgene. Two mice of founder 39 and three mice of founder 31 were used in this experiment.

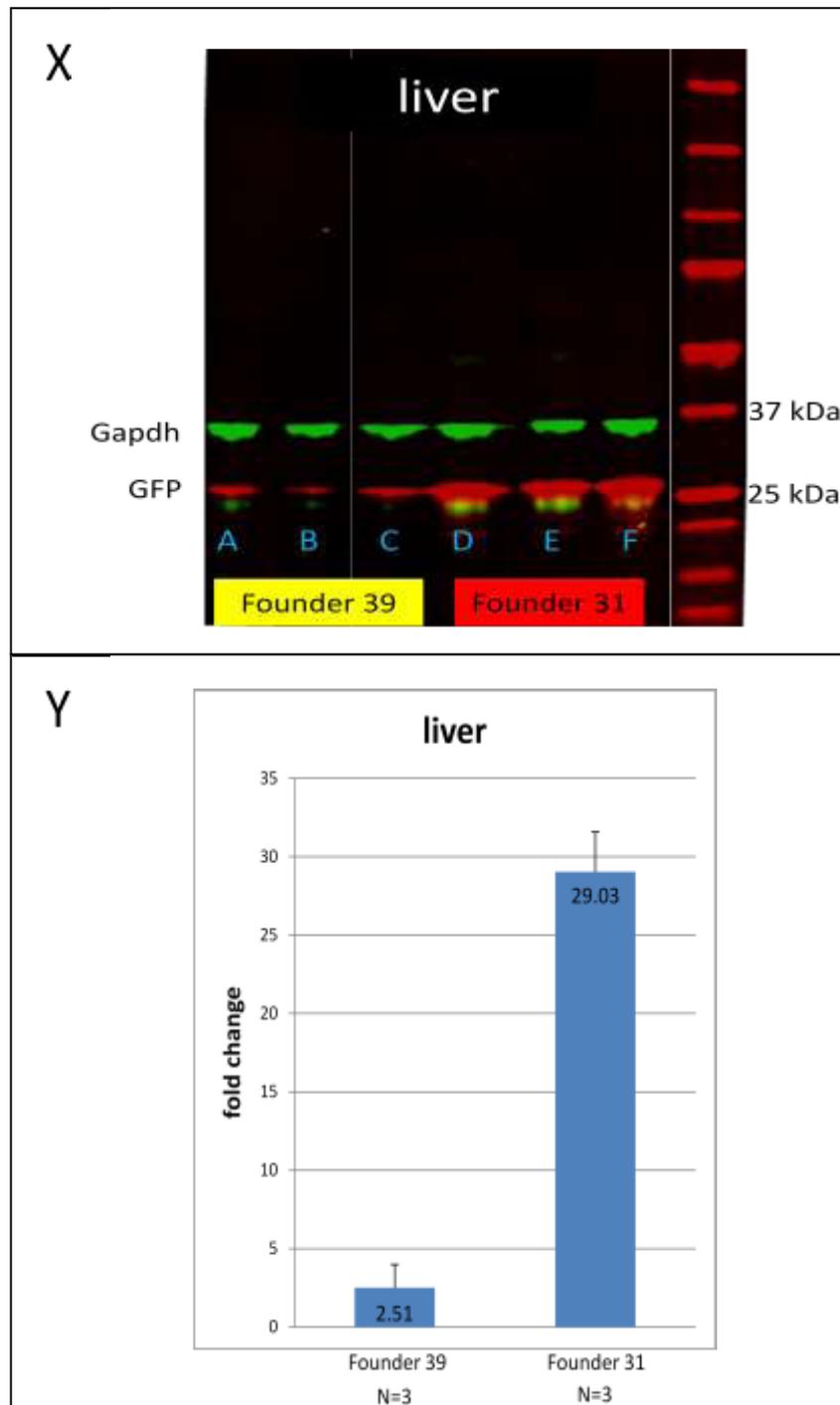


Figure 11. Western blot analysis for GFP in the liver comparing founder 31 and 39 of Tet-on-99CGG-eGFP. Figure X is the result of Western blot. A, B and C are mice from founder 39. D, E and F are from founder 31. The red bands are the GFP signal. The green bands are signal of Gapdh protein which is used as internal control. Founder 31 gave much stronger GFP signal than founder 39. The levels of GFP protein can be seen in figure Y. The GFP levels of founder 31 were almost twelve times higher than founder 39.

5.8. Formation of ubiquitin intranuclear inclusion in the Tet-on-99CGG-eGFP/PrP-rtTA bigenic mice

The Tet-on-99CGG-eGFP/PrP-rtTA bigenic mice from a breeding between the best of each of the founders, then were subjected for ubiquitin-positive intranuclear inclusions assessment. These bigenic mice were treated with dox in drinking water for 4 weeks, and were sacrificed to collect the brains for the neuropathological studies. Immunohistochemistry was performed to check the GFP expression as well as the presence of ubiquitin-positive inclusions formation in the brain slices. Immunohistochemistry for GFP showed that the transgene was expressed by the presence of GFP staining in the brains (Figure 12). On the other hand, immunohistochemistry for ubiquitin did not detect any ubiquitin-positive inclusions (data not shown). It seemed that the expression of the expanded premutation CGG induced by dox in drinking for 4 weeks was not enough to produce the neuropathological hallmark of FXTAS, ubiquitin-positive intranuclear inclusion.

In addition, the exposure of the toxic CGG to the bigenic mice was lengthened by treating with dox in drinking water for longer period, 12 and 16 weeks, and sacrificed the mice to observe the presence of ubiquitin-positive intranuclear inclusions as well as the GFP expression by immunohistochemistry. immunohistochemistry for GFP showed that more cells were stained on 12 & 16 weeks of dox than 4 weeks. It seemed the longer period of dox treatment, the more cells were exposed to the inducer, and hence the more cells would express the transgene. In this experiment the data of GFP RNA and protein levels by RT-

Q-PCR and Western blot were gained. Although the expression of the transgene was limited to some parts of the brain, the GFP signal could still be detected through RT-Q-PCR and Western blot (Figure 13 & 14). The RT-Q-PCR showed increase levels of transgene transcript by the time of dox exposure. The 12 weeks of dox treatment gave the highest levels of RNA instead of the 16 weeks of treatment. Using the Western blot, the expression of GFP could be seen by the presence of the GFP bands. GFP levels tended to increase by the time of dox treatment except for the 12 weeks of treatment which gave lower levels than 4 weeks of treatment.

Ubiquitin immunohistochemistry showed the presence of ubiquitin-positive inclusions in the brain slices of The Tet-on-99CGG-eGFP/PrP-rtTA bigenic mice after 12 weeks of dox in drinking water treatment. While in the bigenic mice with 16 weeks of dox treatment, even more ubiquitin-positive inclusions were observed than after the 12 weeks of treatment (Figure 15). The ubiquitin-positive inclusions were found mainly in hippocampus and cerebellum, comparable with the areas where GFP expression was mainly found (Figure 12). It has proven that the inducible transgenic system in this bigenic line has succeeded to produce FXTAS neuropathology hallmark, the ubiquitin-positive intranuclear inclusions. This finding also gave information about the period of dox in drinking water treatment to this bigenic line which was able to form ubiquitin-positive inclusions.

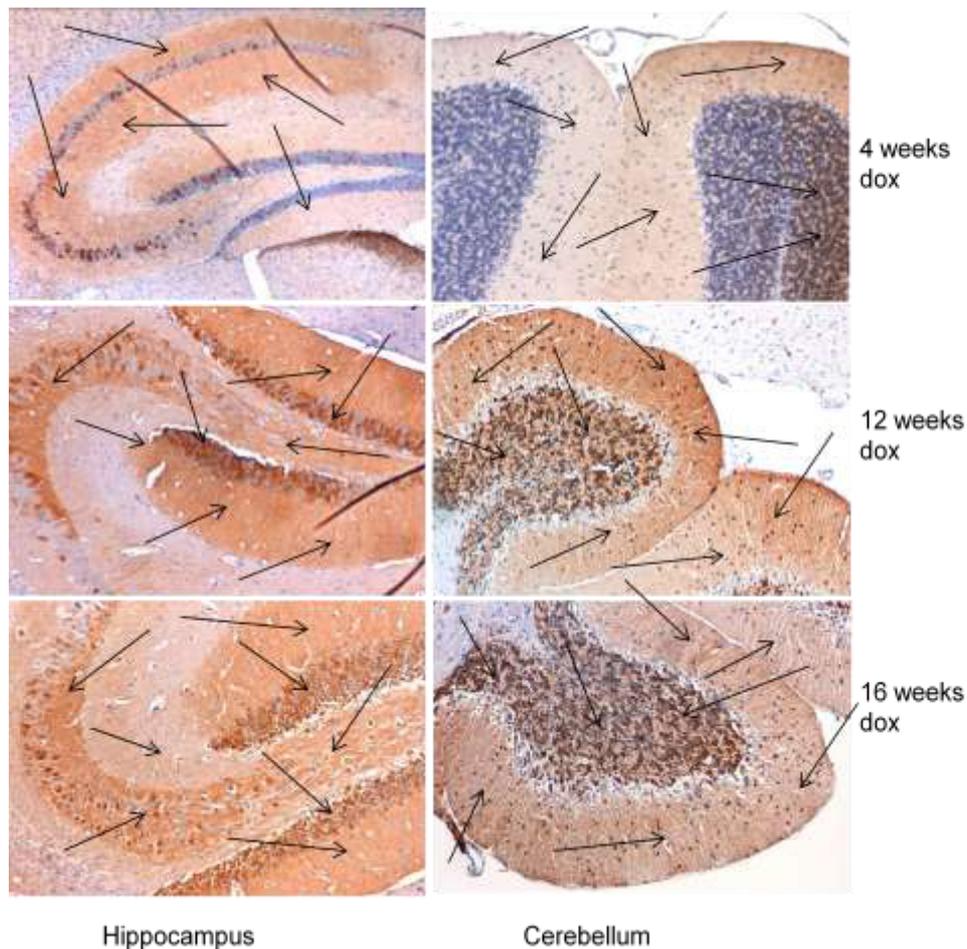


Figure 12. Immunohistochemistry for GFP in the brain slices of bigenic mice Tet-on-99CGG-eGFP/PrP-rtTA after dox treatment. Positive results were indicated with the brown color staining (arrow), which means the tissues expressed the transgene. These bigenic mice expressed the GFP in the brain mainly in hippocampus and cerebellum. 100X magnification.

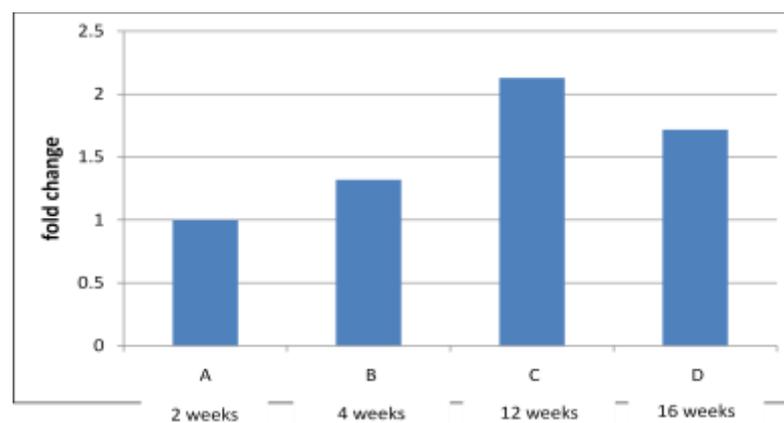


Figure 13. GFP RNA levels in the brain of bigenic mice Tet-on-99CGG-eGFP/PrP-rtTA after dox treatments. A is the bigenic mice with two weeks of dox treatment from the previous experiment. B, C and D are the bigenic mice after 4, 12 and 16 weeks of dox treatments respectively. The GFP expression could also be detected in RNA form by RT-Q-PCR. The levels of GFP in RNA form tended to increase by the longer period of dox treatment.

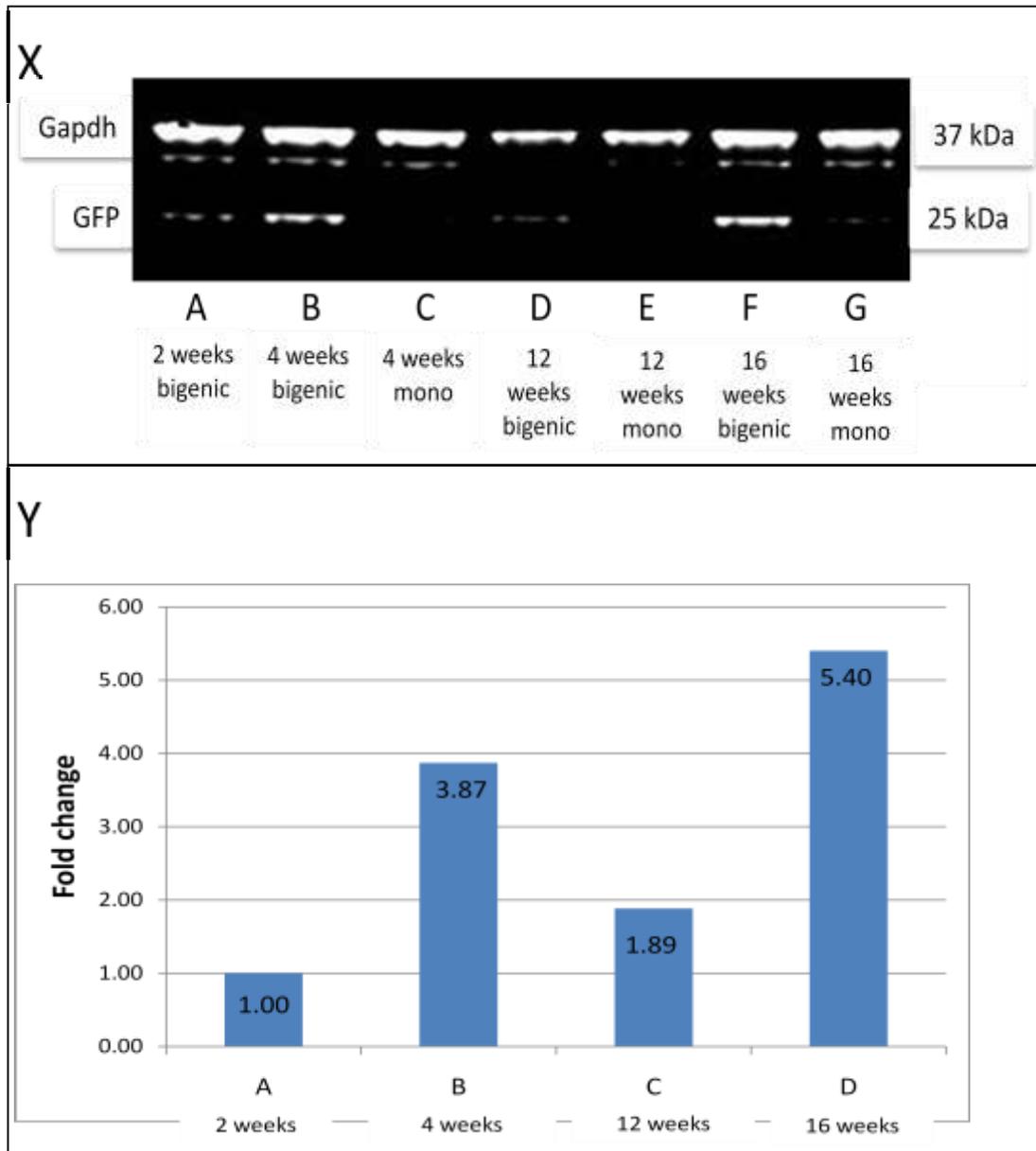


Figure 14. Western blot for GFP of bigenic mice Tet-on-99CGG-eGFP/PrP-rtTA after different time points of dox treatment. The transgene expression could also be detected by Western blot. The Western blot (figure X) showed the presence of transgene expression, indicated by the appearance of GFP bands at 27 kDa after incubating with antibody against GFP. Gapdh was used as loading control which is shown with bands at 37 kDa. Figure Y is the GFP expression levels based on the Western blot results. A is the bigenic mice with 2 weeks of dox treatment from the previous experiment. B, C and D are the bigenic mice after 4, 12 and 16 weeks of dox treatments respectively. The GFP protein levels tended to increase with longer period of dox induction.

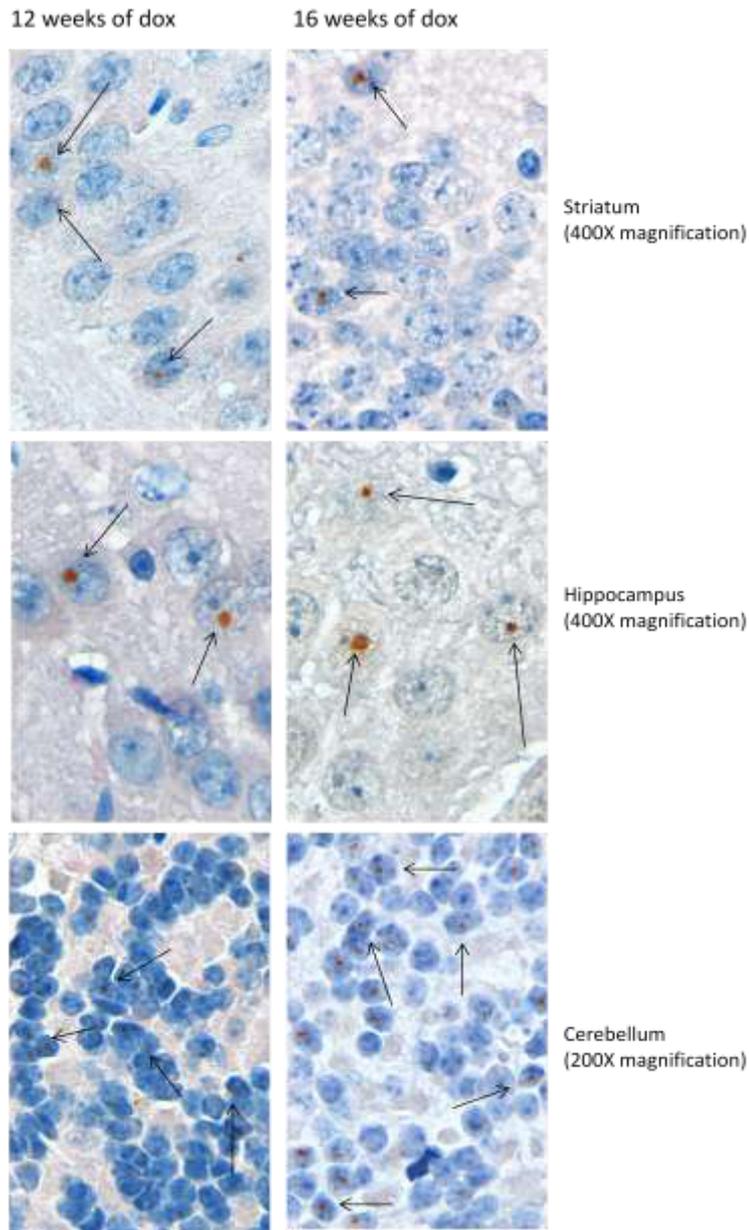


Figure 15. Immunohistochemistry of ubiquitin in the brain slices of bigenic mice Tet-on-99CGG-eGFP/PrP-rtTA with 12 and 16 weeks of dox treatment. The presences of ubiquitin-positive intranuclear inclusions were indicated with the brown round spot (arrows). The ubiquitin inclusions were formed after 12 weeks of dox induction/transgene expression. Weeks 16th of dox induction/transgene expression gave more ubiquitin inclusions than weeks 12th. Four weeks of dox induction/transgene expression was not enough to form ubiquitin inclusions. Ubiquitin inclusions were mainly found in neurons cells of hippocampus, cerebellum, and striatum.