

Using Conditional Autophagy Inhibition in Mice to Study Neurological Disorders

University Honors Capstone

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Spring 2018

Abstract

Introduction: Autophagy is a cellular mechanism involved in the clearance of aggregated cytosolic proteins, and has been studied in relation to neurodegenerative disorders such as Parkinson's disease and depression. This project aims to create a mouse model of neurodegenerative disease and affective disorder through a conditional, targeted knockout of the autophagy associated *Atg 5* gene. **Methods:** Mice carrying a floxed *Atg 5* allele were cross-bred with transgenic mice carrying a Cre-recombinase enzyme driven off an inducible, neuronally expressed promoter. Resultant progeny were genetically selected to carry both the floxed *Atg 5* allele and the Cre recombinase, thus allowing induction of gene deletion to occur at a defined age. Conditional knockout mice were induced at 6 weeks of age and assessed via a battery of behavioral and physical tests, including open field, beam traversal, grip strength, RotaRod performance test, gait analysis, clasping analysis, black and white box, cookie preference, sweet solution preference test, amphetamine induced open field, and replenishment of L-Dopa.

Results: Preliminary data suggests that deletion of the *Atg 5* gene results in the development of a manic phenotype revealed by increased activity observed in the open field test within 5 weeks of gene deletion. No evidence of motor impairments was noted at this time point post induction.

However, at 9 months post-induction a significant neurological phenotype emerged with knockout animals continuing to demonstrate increased open field activity, but now also demonstrating reduced ability to remain on the spinning RotaRod, reduced grip strength,

abnormal gait, uncontrolled tremor, and abnormal clasping reflex. **Discussion:** Deletion of the *Atg 5* gene results in age-dependent development of behavioral and physical changes associated with the phenotype of "mania" and "neurodegeneration". This novel mouse model is amenable to screening studies for the discovery of novel small molecule drugs for the treatment of psychiatric and neurological disease.

Introduction

Autophagy is a cellular mechanism in which a cell essentially digests parts of itself as a protection from harmful molecules. Autophagy is involved in the process of clearing unwanted molecules from the cytoplasm, in order to prevent harmful accumulations within a cell. When molecules such as proteins aggregate and accumulate within the cytoplasm of a neuron, neurodegenerative or affective disorders, such as Parkinson's disease, depression, and mania can result (Yang & Klionsky 2009). In these disorders, autophagy is one of the mechanisms that does not occur properly, preventing the clearance of aggregated proteins, which can overwhelm the neuron until it is dysfunctional.

Additionally, it has been suggested that autophagy may be involved in the mechanism of action of antidepressants and mood-stabilizing drugs. Previous data from a collaborator's lab demonstrated that repeated administration of compounds that enhance autophagy via different pathways, including rapamycin, trehalose and nicardipine result in mood stabilizing-like effects and in changes in autophagy-related protein levels indicative of enhanced autophagy in the frontal cortex of mice (Cleary et al. 2008; Kara et al. 2013). Therefore, by creating a mouse model in which autophagy is inhibited, the relationship between affective disorders and autophagy can be further explored.

In this particular study, *Atg5*, a gene required for autophagy, was knocked out using a cre-recombinase system. A Western blot was performed by another member of the lab to confirm that the knockout had successfully occurred. In the bulk of this study, behavioral tests were performed in order to assess manic or Parkinsonian symptoms in this mice. These tests were quantified when possible, and qualitatively assessed when statistical analysis was not possible. Based on our previous cohorts of knockout mice, as well as pharmacological data, we

hypothesized that conditional deletion of *Atg5* will result in behavioral changes related to affective-like pathology as well as Parkinsonian symptoms. In previous cohorts, some sex differences in severity of knockout phenotype have been observed, so these were examined further in this study. These mice may be used in future research as a mouse model for drug delivery studies related to the treatment of affective and neurodegenerative disorders.

Materials and Methods:

Creation of *Atg5* Knockout Mice and Induction

All studies and protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee prior to beginning research. Mice carrying a floxed *Atg5* allele were cross-bred with transgenic mice carrying a Cre-recombinase enzyme driven off an inducible, neuronally expressed promoter. Resultant progeny were genetically selected to carry both the floxed *Atg5* allele and the Cre recombinase. The breeding scheme is shown in Figure 1. The combination of a floxed *Atg5* allele with Cre recombinase allowed induction of gene deletion to occur at a defined age using tamoxifen. The concentration of *Atg5* protein in the cerebral cortex of knockouts and controls was determined in an experiment by a different member of the lab to ensure the gene had successfully been knocked out. Behavioral tests were performed after induction, and again around 9 months post-induction.

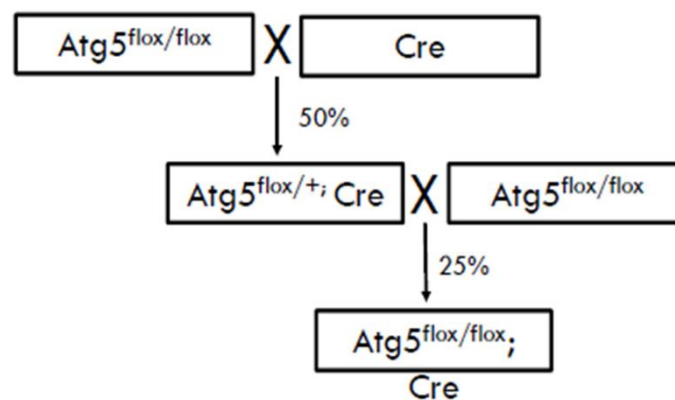


Figure 1. Breeding Scheme Summary. Creating an inducible knockout *Atg5* gene in a mouse model.

Open Field Test

Mice were placed in a novel environment, clear bins, and filmed for 30 minutes to assess exploratory activity. The setup is shown in Figure 2. Ten minutes of the filmed time was analyzed using a stopwatch to measure how much time was spent moving.



Figure 2. Open Field Setup. Three mice were placed in labeled bins and filmed to investigate exploratory activity.

Grip Strength

Mice were placed on a metal grid and allowed to get their footing. After a few seconds, the grid was subsequently flipped upside down. Time for the mice to fall onto the pad below was recorded. After 60 seconds, the test was ended if the mice had not already fallen. Three replicates of this test per mouse were performed.

Rotarod Performance Test

Mice were placed on a Rotamex machine, which has a rotating bar that increases in RPM over a set period of time, as seen in Figure 3. In this test, the Rotamex increased 1 RPM every 3 seconds, maxing out at 20 RPM. Mice had to increase their speed as well as keep their grip in order to stay on the rotating rod. Time for the mice to fall off the rotarod, as well as the RPM at

the time of their fall was recorded. Four replicates of this test per mouse were performed over the course of a week.



Figure 3. Rotamex Machine for Assessing Rotarod Performance. Four mice, one in each section, were placed on the Rotamex in each test.

Gait Analysis

Mice were trained to run down a walkway created within a light/dark box. Once trained, white paper was laid down on the length of that walkway. The feet of the mice were painted, with the hind feet blue and the front feet red. The mice were placed on the walkway, and their gait pattern was imprinted on the paper via the paint. The gait analysis papers were qualitatively analyzed to visualize gait patterns indicating Parkinsonian phenotypes. This test was performed on an earlier cohort of *Atg5* mice.

Clasping Analysis

Over the course of other behavioral tests, it emerged that some knockout mice displayed an abnormal clasping reflex when held by their tails. In order to investigate this more uniformly,

each mouse was held by their tail for 30 seconds and assessed qualitatively for the presence or absence of this clasping reflex.

Statistical Analysis of Results

G-tests were performed on the data to determine any outliers. One-tailed student t-tests were performed to compare wild type to knockout overall. P-values less than 0.05 were considered significant. T-tests were also performed on the data for males and females separately, to examine differences in phenotype within the sexes. The final two tests – the gait and clasping analyses – were qualitative tests, and therefore not analyzed using statistics.

Results

Concentration of *Atg5* protein in the cerebral cortex of the floxed-Cre mice was significantly less than the concentration within control mice. Control mice had 2.2 times as much *Atg5* present in their cortex than the knockout mice, as seen in Figure 4. This indicates that an observable molecular difference between the knockouts and controls existed. Behaviorally, no observable phenotype was observed in the knockouts compared to the controls immediately post-induction, but when reassessed after 9 months, some differences emerged.

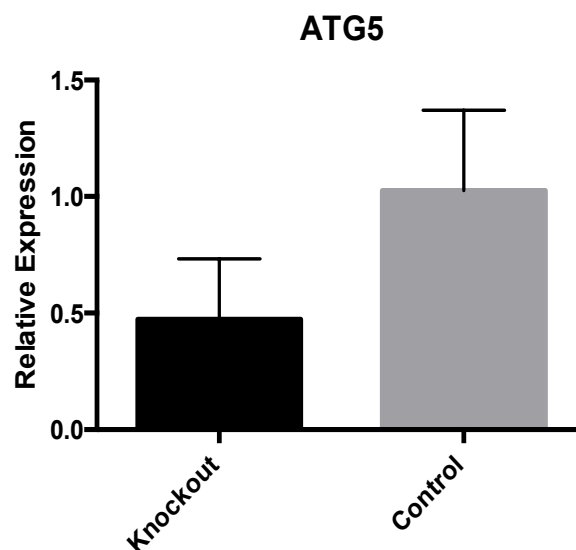


Figure 4. Relative *Atg5* protein concentration in the cortex. *Atg5* protein is 2.2 fold higher in control mice cortex. [p=0.046]

In the open field test, there were no conclusive differences found between the knockouts and the controls. In the whole sample, knockout mice displayed virtually no difference in activity compared to the controls, as seen in Figure 5. The p-value for differences in the whole sample was 0.3894 (Fig. 5). When the mice were analyzed by sex, the male wild types showed more overall activity than the knockout group, as seen in Figure 6. However, this was not a significant difference ($p = 0.1256$). Figure 7 shows the results for the females. On average, knockouts showed higher activity, but again, this was not significant ($p = 0.1075$).

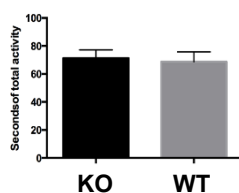


Figure 5. Comparison of Open Field Total Activity for Entire Sample. On average, there was very little difference in overall activity between the knockouts and wild-types. $p = 0.3894$

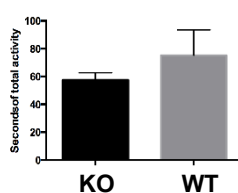


Figure 6. Comparison of Open Field Total Activity for Males. Male wild-type mice showed more overall activity than the knockout mice on average, but not significantly. $p = 0.1256$

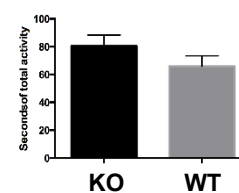


Figure 7. Comparison of Open Field Total Activity for Females. Female *Atg5* knockout mice showed more average activity than the wild-type mice, but not significantly. $p = 0.1075$

There was no significant difference in Rotarod performance between knockouts and wild-type mice in the overall sample, as seen in Figure 8. There was a slight trend in which the wild-types could stay on somewhat longer, but it was not significant ($p = 0.1265$). However, for the males, there was a significant difference in performance between wild-type mice and knockouts ($p = 0.0011$). As can be seen in Figure 9, male wild-types stayed on the Rotarod significantly longer than the knockouts. There was no such difference for the females, as seen in Figure 10. In fact, the female knockouts, on average, stayed on the Rotarod slightly longer than the wild-types, but it was not a significant trend ($p = 0.198$).

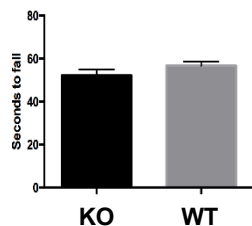


Figure 8. Comparison of Rotarod Performance for Entire Sample. On average, wild-type mice were able to stay on the Rotarod longer than knockouts, but not significantly. $p = 0.1265$

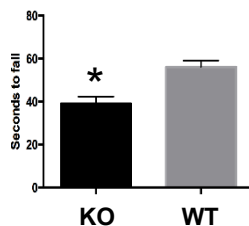


Figure 9. Comparison of Rotarod Performance of Males. Wild-type males were able to stay on the Rotarod significantly longer than knockouts. $p = 0.0011$

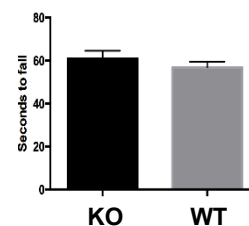


Figure 10. Comparison of Rotarod Performance of Females. Knockout females were able to stay on the Rotarod for an average time longer than the wild-type females. $p = 0.198$

In the grip strength test, there was no significant difference between the wild-types and knockouts in the overall sample ($p = 0.3747$). As seen in Figure 11, there was virtually no difference in average amounts of time to fall between the two groups, but a slightly longer average time to fall for the wild-types. Figure 12 shows the results for the males. For male mice, knockouts were able to hold on slightly longer on average, but this was not a significant difference ($p = 0.3728$). For females, the opposite trend was shown, with the female wild-types gripping the grid slightly longer than the knockouts, as seen in Figure 13. However, this was also not a significant trend ($p = 0.2843$).

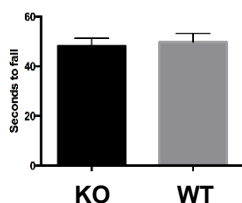


Figure 11. Comparison of Grip Strength, Represented by Average Time to Fall, for Entire Sample. Wild-type mice were able to hold on marginally longer than knockouts, but this is not significant. $p = 0.3747$

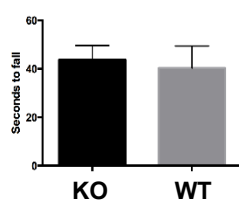


Figure 12. Comparison of Grip Strength for Males. On average, knockout males were able to hold on longer than wild-type males, but not significantly. $p = 0.3728$

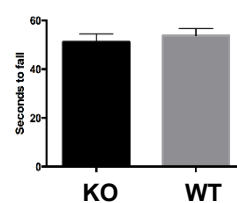


Figure 13. Comparison of Grip Strength for Females Wild-type females were generally able to hold on longer than knockouts, but not significantly. $p = 0.2843$

For the qualitative gait analysis, certain patterns in gait could be seen visually in knockout mice severely affected with the phenotype when compared to controls or mildly affected knockouts. Representative gait analysis sheets of a wild-type mouse and a knockout mouse displaying the phenotype are shown in Figure 14. The wild-type gait analysis is orderly, and footprints are clearly defined (Figure 14). However, in the knockout, the footprints are disordered and visible dragging of the hind feet has occurred (Figure 14).



Figure 14. Gait Analysis of a Representative Wild Type and Severely Affected Knockout Mouse. Knockout displayed splayed hind legs and an abnormal walking pattern. Both mice were female, and from an earlier cohort.

When knockout mice were held by their tails as demonstrated in figure 15, some of them displayed an obvious abnormal clasp reflex that was not present in the wild types. In this reflex, the mice would tightly clutch their legs inward instead of keeping them splayed apart like the wild-types (Figure 15). Some of the mice would tightly clasp both hind and front legs, while many would only clasp their hind legs. Others would only clasp one of their legs. There was a spectrum of severity in the display of this phenotype, with some mice displaying a strongly abnormal, obvious reflex, and others showing a mildly abnormal reflex. The results of these qualitative clasp assessments are shown in Table 1. Only the knockouts displayed any of these clasp reflexes, and the male knockouts showed the obvious phenotype more often than the females (Table 1). Female knockouts tended to display the mildly abnormal clasp reflex or a normal phenotype rather than the strong phenotype. Male knockouts showed the strongly abnormal phenotype more often than the mild or normal phenotype (Table 1).

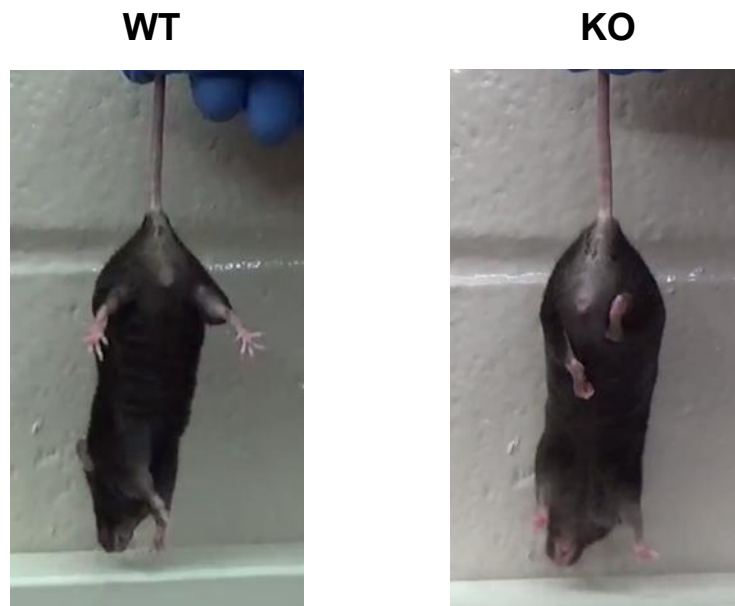


Figure 15. Comparison of Wild-Type Mouse with Male *Atg5* Knockout Demonstrating Abnormal Clasp Reflex. Knockout mice displayed mild to severe clasp abnormalities when held by their tails, as compared to wild type mice.

Table 1. Clasping Analysis Results

	WT Males	WT Females	KO Males	KO Females
Normal Phenotype	3	8	1	4
Mild Abnormal Phenotype	0	0	2	4
Strong Abnormal Phenotype	0	0	3	1
Total Number	3	8	6	9

Overall, no significant patterns in data have emerged that have been consistent across the entire sample. The knockouts showed no significant differences in open field activity or grip strength when compared to the control. However, male knockouts did display a neurological phenotype, with significantly reduced Rotarod performance, as well as abnormal clasping reflexes. These results will be further discussed below.

Discussion

Although there was only one significant difference between the knockouts and wild-types in these behavioral tests, this can still shed light on areas to be explored in further research. Behavioral tests always have the potential to be slightly subjective, even with the best efforts to make them as quantitative as possible. Also, neurodegeneration and affective disorders affect each individual differently, so it is entirely possible that some mice were simply at different stages even though they had been induced at the same age. Additionally, this colony was started multiple cohorts ago, and there is always the possibility that later cohorts of mice were genotyped incorrectly.

In the open field test, it was expected that a phenotype of *Atg5*-inhibited mania would be demonstrated by increased levels of activity in the knockouts. If the opposite had occurred, it

could have been evidence of Parkinsonian symptoms interfering with movement. However, there was no significant difference in the overall sample, and the males and females showed conflicting trends. Therefore, no major conclusions could be drawn based on the results of the open field test.

Similarly, the grip strength test was inconclusive in demonstrating differences between wild-types and knockouts. None of the analyses showed any significant differences, and the knockouts and wild-types performed very similarly across all three. It was expected that neurodegeneration would lead to decreased grip strength, especially in light of the abnormal clasping reflexes displayed in the knockouts. However, the results did not support this hypothesis.

The Rotarod was the only test in which a significantly different result was observed, in that the male knockouts demonstrated significantly reduced ability to stay on the Rotarod compared to controls. This was not true of the overall sample, or of the females, but this significant result does provide some support by quantitatively demonstrating one difference between the two groups. Although the Rotarod did not provide consistently different results, it opens up the interesting possibility of sex differences in this colony of mice.

With the two qualitative measures, a phenotype could be observed in severely affected mice. The female mice in the gait analysis were from an earlier cohort, and were therefore not part of the group that underwent behavioral testing. Their gait analyses were included in order to provide a representative sample of how *Atg5* knockout mice can demonstrate abnormal gait patterns as well as leg dragging, indicating Parkinsonian symptoms. In the future, it would be an interesting avenue of research to explore a quantitative gait analysis, as these do exist. Knockout mice displayed various severity of abnormal clasping reflexes (Figure 15). The absence of this

clasp reflex in wild-type mice indicates that this clasp reflex could be a possible identifier of neurodegeneration. Obviously, this is a qualitative test, and therefore subjective, but it will be interesting going forward to examine this clasp reflex in later cohorts of these mice.

There appear to be some observable sex differences in severity of neurological phenotype. Males showed significantly reduced Rotarod performance, as well as a strong abnormal clasp reflex, while females showed insignificantly heightened Rotarod performance, and mild or no abnormal clasping reflexes. This suggests that sex differences exist in this mouse model of neurological disorders, and should be explored more in further research. It is already known that men are more likely to be affected with Parkinson's, a neurodegenerative disorder, so it is possible that this is the case with these mice as well. Interestingly, the knockout females in an earlier cohort of mice, from which the gait analyses were taken (Figure 14), were more severely affected with the neurological phenotype than the males.

Conclusion and Implications for the Field

In the future, these tests should be repeated with later cohorts of mice. The mice should also be genotyped again to ensure that the colony is correctly categorized. If possible, the qualitative tests, particularly the gait analysis, could be modified to become a quantitative measure. Although the results of these behavioral tests were mostly inconclusive, some mice are clearly exhibiting a neurological phenotype 9 months post-induction, and this research provides a jumping off point for further exploration with this colony. The ultimate goal of this research study is to successfully create a mouse model of neurological disorders such as mania and Parkinson's. With the creation of this mouse model, drug delivery research can be conducted, with the goal to test new and existing treatments for neurodegenerative and affective disorders.

References

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Acknowledgements

I would like to thank Grant Anderson for being my faculty mentor and providing guidance throughout the process. I would also like to thank Carl Anderson, another lab member, for teaching me how to perform many of the behavioral tests. Thank you to Carrie Kulibert and Rebecca Sauter for providing moral and proofreading support while writing this paper.

The Anderson lab would like to thank the University of Minnesota, College of Pharmacy for providing the GAP Grant, as well as the United States-Israel Binational Science Foundation for providing funding. We would also like to thank Haim Einat, our collaborator, who provides assistance with statistics from Tel Aviv-Yaffo Academic College, Israel.