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Investigating Aggression in Huntington Disease

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INVESTIGATING AGGRESSION IN HUNTINGTON DISEASE

by

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ABSTRACT

Huntington Disease (HD) is a neurodegenerative disorder that is caused by a CAG trinucleotide repeat expansion in the huntingtin gene. The onset of the disease is defined by the presence of motor deficits, such as chorea. However, cognitive and psychiatric symptoms often develop before motor onset and typically have a larger impact on patient quality of life. Psychiatric symptoms include depression, anxiety, and OCD, but also aggression and irritability, which have been comparatively understudied due to stigma. Currently, treatments to modify these behaviors in premanifest HD patients are not consistently effective and often have side effects, creating a need for research into these psychiatric disturbances. Our lab has observed increased-aggression in our humanized HD mouse model (Hu97/18) during routine handling that is not present in our knock-in HD mouse model (Q175FDN). In this study, we seek to quantify the aggressive behavior exhibited by these two mouse models and determine the neurological basis for these observed behavioral differences. From this analysis, we seek to identify potential therapeutic targets for modulating aggressive behavior in mice, which could lead to the development of therapies that reduce the aggressive behavioral symptoms experienced by HD patients.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

β-NADPH = Beta-nicotinamide adenine dinucleotide phosphate

C57BL/6 = Black 6 mouse

FVB/N = FVB, wild-type mice

HD = Huntington Disease

HTT = huntingtin

Hu18/18 = humanized control mouse model

Hu97/18 = humanized HD mouse model

mtHTT = mutant huntingtin

NHC = neutral homecage

PBS = phosphate buffered saline

Q175FDN = knock-in HD mouse model

RI = resident-intruder

SEM = standard error of measurement

UHDRS = unified Huntington's disease rating scale

VMH = ventromedial hypothalamus

WT = wild type

CHAPTER ONE: INTRODUCTION

Huntington Disease

Huntington Disease (HD) is a neurodegenerative disorder caused by a CAG repeat expansion in the huntingtin (*HTT*) gene of greater than 35 [1]. Individuals with 36-39 CAG repeats have reduced penetrance of HD, meaning they may never develop HD symptoms. However, 40 or more CAG repeats is fully penetrant and affected individuals will develop HD symptoms. Clinical diagnosis of HD is based on the presence of the CAG repeat expansion of 36 or more repeats combined with motor performance deficits on the Unified Huntington's Disease Ratings Scale (UHDRS), which is a standardized test used to assess motor and cognitive function in HD patients. However, some HD patients develop cognitive and psychiatric symptoms prior to motor symptoms. Psychiatric symptoms include depression, anxiety, aggression and irritability [2, 3], and of these symptoms, aggression and irritability have been comparatively understudied due to stigma. However, aggression and irritability have an extremely negative impact on the quality of life for patients and caregivers, making it important to investigate and characterize these aspects of HD [4].

Aggressive behavior is typically categorized as either pre-meditated or impulsive aggression. Pre-mediated aggression is characterized by well-planned behavior that is not associated with autonomic arousal or provocation [5]. However, impulsive aggression, also called reactive aggression, is characterized by unplanned, emotionally-driven aggressive episodes associated with autonomic arousal. Impulsive aggression becomes pathological when the aggressive response is exaggerated compared to the initial provocation [5]. Previous studies

seeking to characterize aggression displayed by HD patients found that verbal outbursts and being easily irritated or impatient are common expressions of aggressive behavior [6, 7]. Additionally, it appears that the triggers for these aggressive episodes may be emotion-based, such as frustration from changes in routine or requiring assistance from a caregiver, but aggressive episodes may also appear without a clear antecedent event or trigger [8]. Based on these previous findings, it is suggested that aggression in HD is pathological impulsive aggression.

Neurological Basis for Aggression in HD

Previous studies have found that reduction in volume of the amygdala and ventromedial hypothalamus may occur before HD onset [9-12]. Additionally, previous studies investigating pathological reactive aggression have found that these structures are associated with aggression [5, 13]. These findings suggest that these neurological structures may mediate pathological aggression experienced by HD patients.

Amygdala

The amygdala is a small structure in the limbic system which mediates emotional responses, such as fear or anger, in response to stimuli. Humans with pathological reactive aggression tend to show hyperactivity of the amygdala [14, 15]. Medial amygdala lesioning in mice reduces some aggressive behaviors, which is consistent with hyperactivation inducing aggressive behavior [16]. However, hypoactivation of the amygdala in pre-HD patients compared to gene-negative controls has been observed [17, 18]. Due to these conflicting findings, the role of the amygdala in mediating HD-related aggression remains unclear.

Ventromedial Hypothalamus

The ventromedial hypothalamus (VMH) is a region in the hypothalamus that mediates multiple functions in metabolism and behavior, but its precise role in aggression appears to be unclear. Previous studies have found that hypothalamic dysfunction results in aggressive behavior [19, 20]. Additionally, lesioning of the posterior or anterior regions of the VMH in rats produces increased aggressive behavior [21]. However, other studies of aggressive behavior in mice have found that inactivation of the VMH via a synthesized drug-receptor pair that hyperpolarizes neurons, specifically in the ventrolateral portion, reduces aggressive behavior [22]. However, the role of the VMH in HD-related aggression has not been explored.

Aggression Differences Observed in HD Model Mice

Our lab has observed that humanized HD model mice (Hu97/18) [23] demonstrate increased aggression compared to the parent FVB/N (FVB) background strain, while knock-in HD model mice (Q175FDN) [24] do not. However, this difference has not been quantified. Additionally, the reason for this observed difference is unknown. Previous studies on aggression have suggested multiple neurological targets may be involved in mediating aggressive behavior in HD, including the amygdala and ventromedial hypothalamus. In this study, we have quantified aggressive behavior exhibited by the two HD mouse models via multiple aggression behavioral paradigms. We are now comparing the neurological targets in both mouse models through brain dissection and immunohistochemistry to further elucidate the potential neurological differences underlying the aggressive behavior differences. This study has furthered our understanding of the

neurological basis of aggression in HD and could lead to the identification of therapeutic targets for modulating aggression and irritability in HD.

CHAPTER TWO: METHODOLOGY

Aggression Assays

In order to quantify the behavioral differences between the Hu97/18 and Q175FDN mice, I have developed neutral home cage and resident-intruder aggression testing paradigms. In both paradigms, an experimental mouse and a standard opponent mouse interact in a standard mouse homecage. The experimental mice are single-housed at least one week prior to testing. Each test lasts a maximum of 10 min, but violent behavior demonstrated by either mouse will end the test prematurely. Each test occurs in the dark phase of a reversed light cycle under red light to reduce anxiety and promote normal behavioral responses when mice are normally awake.

In the neutral homecage (NHC) assay, a standard opponent C57BL/6 (black 6) mouse is introduced into a clean cage simultaneously with the experimental mouse. In the resident-intruder (RI) assay, the test is performed in the homecage of the experimental mouse, and the standard opponent is introduced as an intruder. Hu97/18, Hu18/18, Q175FDN, and FVB mice were assessed in the two aggression testing paradigms using black 6 standard opponents as fighting partners.

Scoring Aggression Assays

Aggressive behavior

Aggressive behavior was quantified based on latency to attack, the frequency of aggressive behaviors, and the time in seconds spent engaging in aggressive behaviors during the trial. Aggressive behaviors include clinch attack, keep down, and chase behavior (Figure 1). This data is totaled and converted into three measures to evaluate the mouse in each test: attack time,

attack frequency, and attack latency. Attack time is calculated by dividing the time spent in aggressive behaviors by the total time of the test to obtain a proportion of the time spent in attack. Attack frequency is calculated by adding the total frequency of aggressive behaviors exhibited in the test. Attack latency represents the amount of time passed before the mouse exhibits a clinch attack.

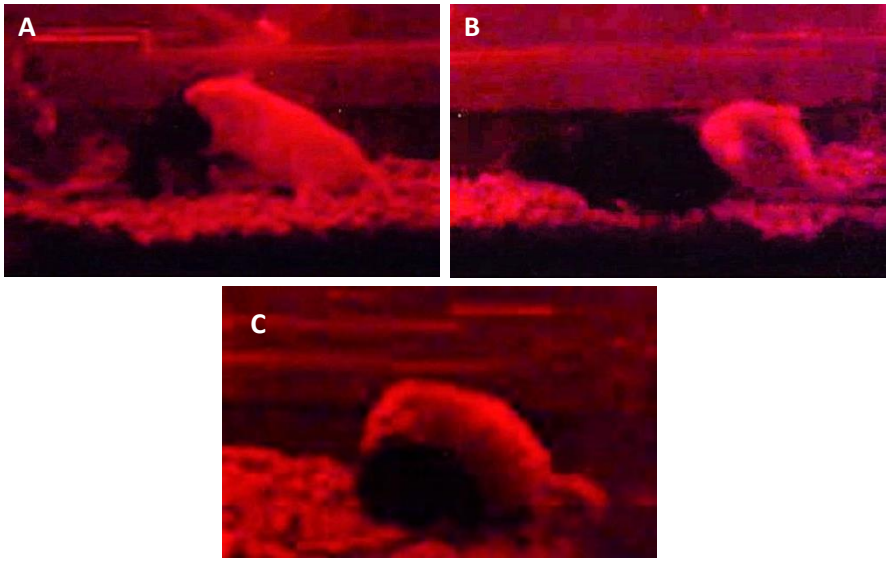


Figure 1: Mice demonstrating different aggressive behaviors used to score the neutral homecage and resident intruder assays **A.** A humanized HD mouse starting a "clinch attack" on the black 6 mouse, which consists of pushing or throwing the mouse and typically starts with a lunge or jump toward the other mouse. **B.** A humanized HD mouse chasing the black 6 mouse. **C.** A humanized HD mouse exhibiting "keep down" behavior, where the humanized HD mouse is holding down the black 6 mouse.

Violent Behavior

Violent behavior is defined as attacking vulnerable areas, including the face, genitals, paws, and belly, or attack behaviors that draw blood. These attacks are forms of escalated aggressive behaviors, and, for the safety of the mice, prematurely end the test. Violent behavior

is scored based on latency to violent behavior, which is the amount of time in seconds before a mouse engages in these behaviors, as well as the percentage of mice who engage in violent behaviors

Generation of Standard Opponents

Standard opponents for the tests are classified as dominant or submissive based on baseline behavior displayed in the neutral home cage testing paradigm using only standard opponents. Dominant behavior is based on the aggressive behavioral assessment described above. Submissive behavior is based on latency to submissive posture, frequency of submissive behavior, and time spent engaging in submissive behaviors. Submissive behaviors include defensive upright posture, submissive posture, and flight (Figure 2).

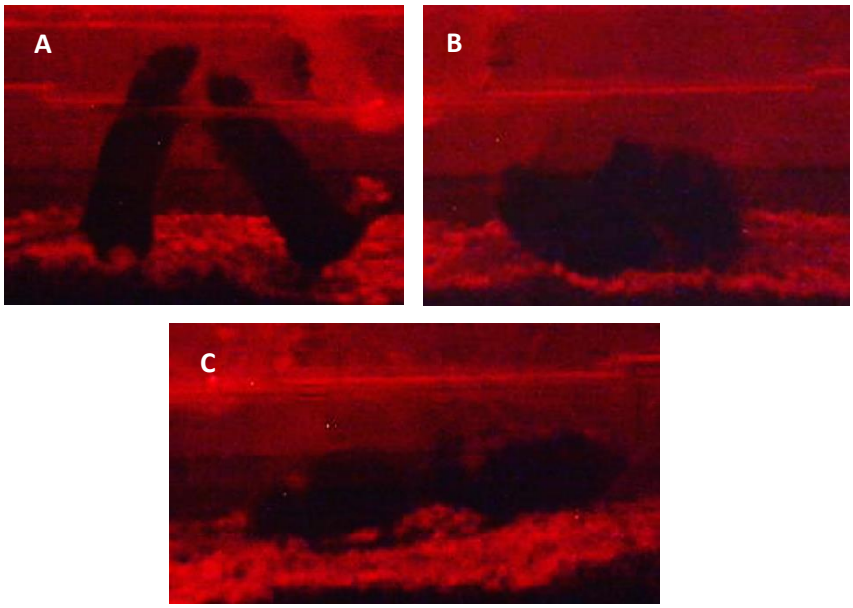


Figure 2: Mice demonstrating different submissive behaviors used to classify standard opponents. A. The black 6 mouse on the left is demonstrating defensive upright posture, which is when the mouse rears on its hind legs in response to an upright posture behavior from another mouse. **B.** The black 6 mouse on the left is exhibiting submissive posture, which is when the mouse remains on the ground during a keep down or clinch attack with their belly exposed and does not attempt to break away from the attack. **C.** The black 6 mouse on the left is fleeing from the other mouse.

Classification as submissive or dominant is based on the aggression behavior score and aggression time scores. The aggression behavior score is the ratio of average dominant behaviors to average submissive behaviors exhibited in the standard opponent aggression testing. The aggression time score is the ratio of time spent exhibiting aggressive behaviors to the total time spent demonstrating any behavior. An aggression behavior score above 1 and an aggression time score above 0.5 indicates the mouse is dominant. Each aggression testing paradigm is completed twice such that each test mouse faces a dominant and submissive standard opponent.

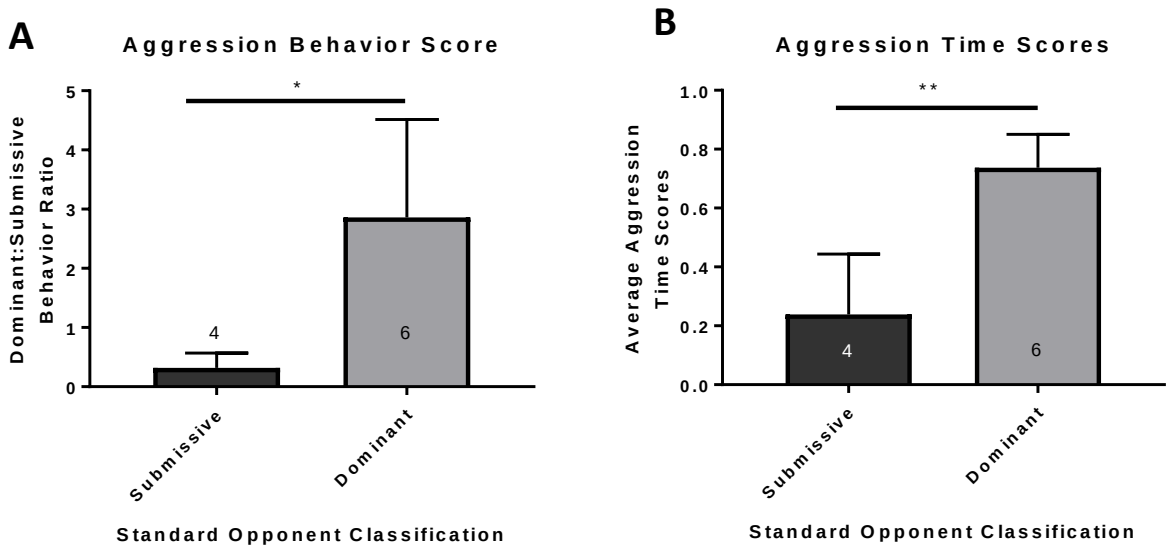


Figure 3: Classification of standard opponents as dominant or submissive. A. Dominant mice exhibited significantly greater aggression behavior scores. **B.** Dominant mice exhibit significantly higher aggression time scores. *=difference between indicated bars by unpaired t-test. * = $p<0.05$, ** = $p<0.01$. Error bars ±SEM

Brain Dissection and Immunohistochemistry

After aggression testing, the mice were perfused and brains were cut by cryostat into 25 µM free-floating coronal sections into a phosphate buffer solution at pH 7.4. In ongoing work, a series of brain sections spanning the amygdala and VMH and spaced 200 µM apart are being stained, and stereological volumetric assessments will be conducted. Using StereoInvestigator software, the perimeters of the desired structures will be outlined. Then, the Cavalieri principle will be utilized using section thickness, spacing, and structure area throughout the series to determine the volume of the structure.

Amygdala Stain

To stain the medial and basomedial amygdaloid structures, we are utilizing NADPH staining, which has been previously shown to stain these structures in rat brains [25]. We will utilize the protocol described in a previous study that sought to stain the central amygdala [26]. In brief, the free-floating sections are placed in a solution of 1mM β-NADPH (Sigma), 0.8 mM nitro blue tetrazolium (Sigma), and 0.06% Triton X-100 in phosphate-buffered saline (PBS). The slides are incubated for 1-2 hours at 37℃. Then, they are rinsed with cold PBS to stop the reaction. The sections are then mounted on slides and dried overnight. Following a serial dehydration and a clearing step utilizing xylenes, the slides are coverslipped using Permount (Fisher) mounting medium.

VMH Stain

To stain for the VMH, are utilizing a Thionin stain that was described in a previous study that conducted volumetric analysis of the VMH in rats [27]. The thionin staining protocol was modified from an online protocol posted by the Kansas Intellectual and Developmental Disabilities Research Center [28]. Briefly, sections are mounted on slides and stained with a Thionin (Sigma) solution (consisting of 18 mL of 0.5% Thionin in distilled water, 180 mL of distilled water, 9 mL of 1.0 M Sodium Acetate solution, and 21 mL 1.0 M Acetic Acid solution at pH 4.3) for 20 minutes at 20℃. Following a serial dehydration and a clearing step utilizing xylenes, the slides are then coverslipped with Permount (Fisher) mounting medium.

CHAPTER THREE: RESULTS

Quantifying behavioral differences in aggression between Hu97/18 and Q175FDN mice

37 mice comprised of 9 Hu87/18, 9 Hu18/18, 10 Q175FDN +/- (heterozygous knock-in: het) and 9 FVB, were utilized for the NHC and RI tasks. Two mice were found dead during the experiment, and one mouse was euthanized for health reasons. This left 34 mice for behavioral testing. After completing behavioral testing, we excluded data from one mouse that exhibited erratic behavior. Additionally, we excluded data points for tests that lasted less than 1 min as well as tests where the standard opponent engaged in violent behavior. After reviewing the standard opponent and mouse behavior for abnormalities, we excluded additional tests that utilized a standard opponent that was deemed hyper-aggressive and a FVB mouse who demonstrated reactive behavior consistent with the naturally occurring FVB seizure disorder [29]. This left data points for 32 mice in the NHC assay and 27 mice in the RI assay.

Neutral Homecage

Hu97/18 and Hu18/18 mice demonstrated similar aggressive behaviors in the neutral homecage assay based on the attack time, attack frequency, and attack latency scores (Figure 4). Both the Hu18/18 and Hu97/18 mice express human *HTT* transgenes and lack mouse Htt. However, Hu18/18 mice express only WT human *HTT*, while Hu97/18 mice express both human WT and mt*HTT*. Hu97/18 exhibit numerous HD-like phenotypes not present in Hu18/18 mice; however, both Hu18/18 and Hu97/18 exhibit metabolic dysfunction and circadian rhythm disruption. Based on this evidence that Hu18/18 mice may not be the most appropriate control for Hu97/18 mice, we decided that the comparison between Hu97/18 and FVB mice was more

relevant to investigate aggressive and non-aggressive HD behaviors. Thus, we have excluded Hu18/18 mice in subsequent analysis.

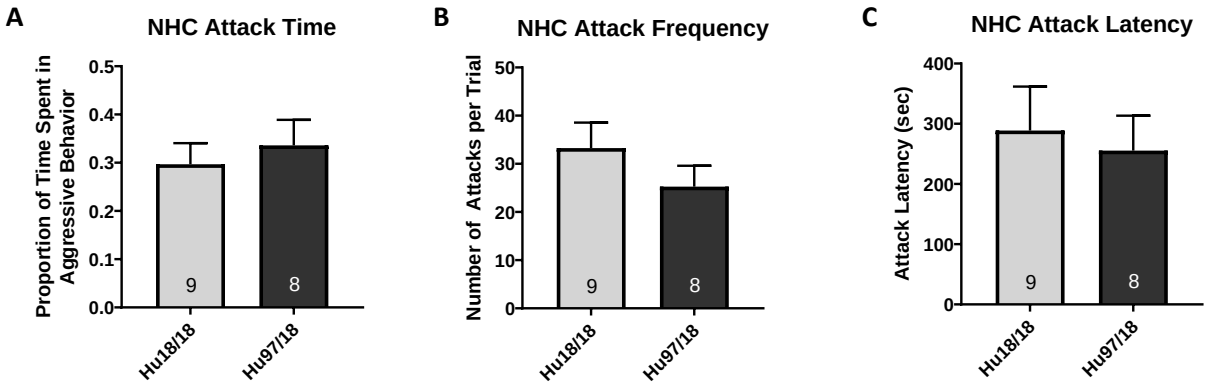
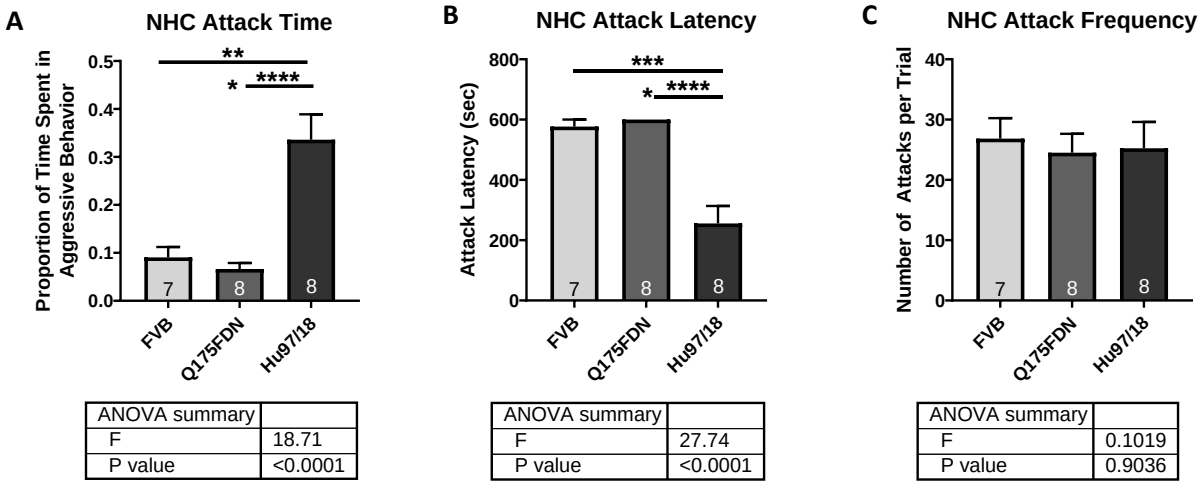


Figure 4: Hu97/18 and Hu18/18 demonstrate similar behaviors during the neutral homecage assay. Mice were tested in the NHC assay under red light in their dark phase with a maximum trial duration of 10 min. No significant differences were observed between Hu18/18 and Hu97/18 mice in all measures of aggression examined.

Compared to FVB mice, Hu97/18 mice exhibited significantly greater proportional attack times while Q175FDN mice did not (Figure 5A, 1 way ANOVA with Turkey's multiple comparisons test compared to FVB, Hu97/18 p=0.0002, Q175FDN p=0.8821). Additionally, Hu97/18 mice exhibited greater proportional attack times compared to Q175FDN mice as well (Figure 5A, 1 way ANOVA with Turkey's multiple comparisons test compared to Q175FDN mice, Hu97/18 p<0.0001). Additionally, Hu97/18 mice exhibited significantly shorter latencies to clinch attack while Q175FDN did not when compared to FVB mice (Figure 5B, Hu97/18 p<0.0001, Q175FDN p=0.9015). Hu97/18 mice also exhibited shorter latencies to clinch attack compared to Q175FDN mice, (Figure 5B, 1 way ANOVA with Turkey's multiple comparisons test compared to Q175FDN mice, Hu97/18 p<0.0001) Taken together, these data suggest

heightened aggression in Hu97/18 mice compared to FVB or Q175FDN mice. Interestingly, Hu97/18, FVB, and Q175FDN mice all displayed similar attack frequencies (Figure 5C, Hu97/18 p=0.9507, Q175FDN p=0.8973), which may indicate similar motivations to engage in aggressive behaviors. However, attack frequency encompasses all forms of aggressive behaviors including violent behaviors, so it may be possible that the observed difference between Hu97/18 mice and the less aggressive Q175FDN and FVB mice may be related to the type of behaviors exhibited rather than the frequency of these behaviors. Thus, the mice would all demonstrate similar motivations in fight behaviors, but Hu97/18 mice may escalate aggressive behaviors and engage in them for longer periods of time per attack.



ANOVA summary	
F	18.71
P value	<0.0001

ANOVA summary	
F	27.74
P value	<0.0001

ANOVA summary	
F	0.1019
P value	0.9036

Figure 5: Hu97/18 mice demonstrate elevated aggression during the neutral homecage assay. Mice were tested in NHC under red light in dark phase with a max trial duration of 10 min. **A.** Hu97/18 mice spent a significantly greater proportion of time engaging in aggressive behaviors compared to FVB mice, while Q17F5DN mice behaved similar to FVB mice. **B.** Hu97/18 mice had significantly shorter attack latencies, latency to engage in a 'clinch' attack, compared to FVB and Q175FDN mice. **C.** Attack frequency was not affected by genotype. *=difference between indicated bars. ***= p<0.001, ****= p<0.0001. Error bars ±SEM

Compared to FVB mice, only 14% of which engaged in violent behaviors, 75% of Hu97/18 mice engaged in violent behaviors (Figure 6A), demonstrating dramatically elevated severity of aggressive behaviors in Hu97/18 mice. Violent behaviors were not observed in Q175FDN mice. Additionally, Hu97/18 mice had a significantly shorter latency to violent behavior compared to FVB mice, while Q175FDN mice did not (Figure 6B, 1 way ANOVA with Turkey's multiple comparisons test compared to FVB, Hu97/18 p=0.0047, Q175FDN p=0.9098). These data demonstrate that unlike Q175FDN HD mice, Hu97/18 humanized HD mice recapitulate HD-related enhanced aggressive behaviors.

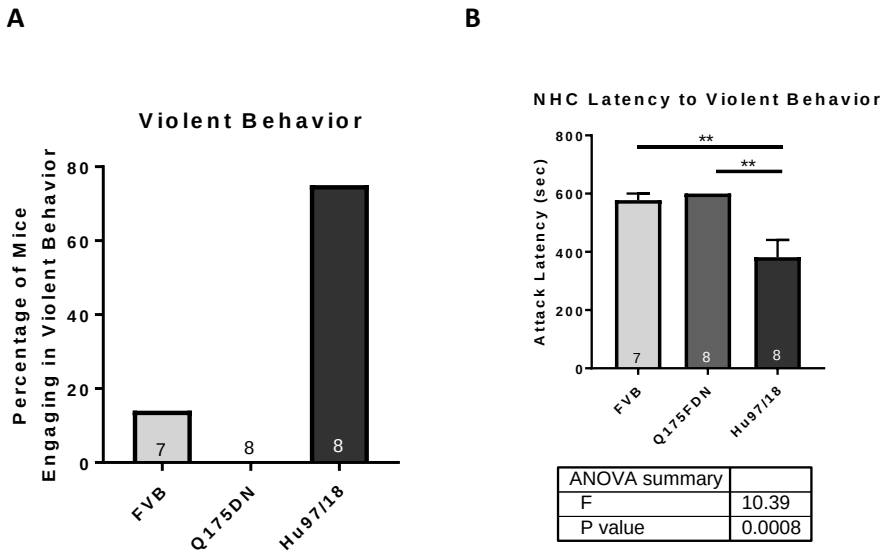


Figure 6: Hu97/18 mice demonstrate increased violent behaviors during the neutral homecage assay. Mice were tested in NHC under red light in dark phase with a max trial duration of 10 min. **A.** A dramatically greater percentage of Hu97/18 mice engaged in violent behaviors compared to FVB and Q175FDN. **B.** Hu97/18 mice had a significantly shorter latency to violent behavior compared to FVB and Q175FDN mice. **= $p<0.01$ between indicated bars. Error bars ±SEM

Resident-Intruder

Compared to FVB mice, Hu97/18 and Q175FDN mice did not exhibit significantly shorter latencies to clinch attack (Figure 7B, Hu97/18 p=0.1740, Q175FDN p=0.3814). However, Hu97/18 mice did exhibit significantly shorter latency to clinch attack compared to Q175FDN mice (Figure 7B, Q175FDN p=0.0150). Interestingly, unlike in the neutral homecage assay, Hu97/18, FVB, and Q175FDN mice all displayed similar attack times (Figure 7A, Hu97/18 p=0.9950, Q175FDN p=0.8595). Additionally, Hu97/18, FVB, and Q175FDN mice all exhibited similar attack frequencies (Figure 7C, Hu97/18 p=0.9039, Q175FDN p=0.3785), which remains consistent with results from the neutral homecage assay. Since similar attack frequencies may indicate that the difference between Hu97/18 mice and the less aggressive Q175FDN and FVB mice may be related to the type and duration of behaviors exhibited rather than the frequency of these behaviors, violent behaviors were assessed again for this assay.

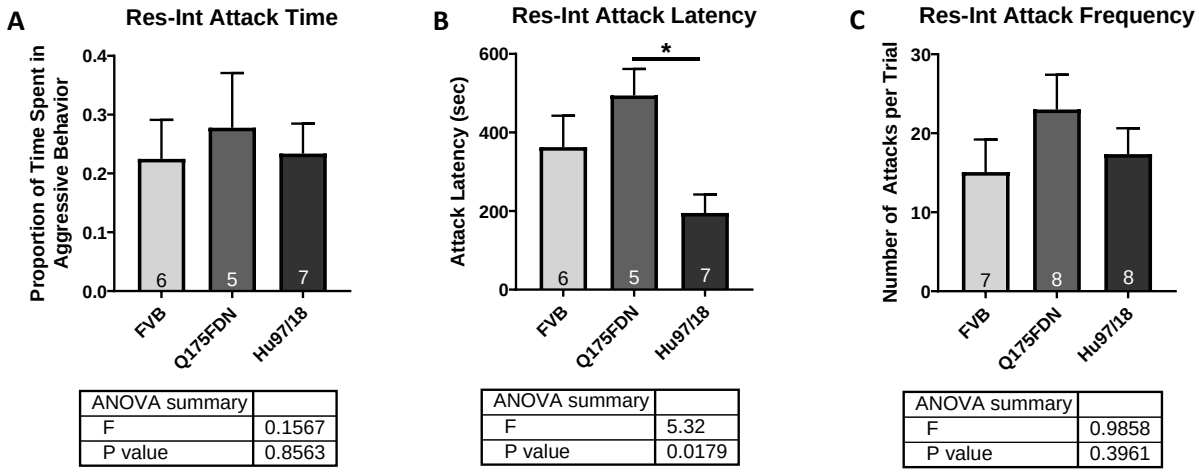


Figure 7: Hu97/18 mice behave similar to FVB mice in the resident-intruder assay. Mice were tested in RI under red light in dark phase with a max trial duration of 10 min. **A.** FVB, Q175FDN, and Hu97/18 mice did not significantly differ in attack time. **B.** Compared to FVB mice, Hu97/18 mice and Q175FDN mice did not differ in attack latency, the latency to engage in a 'clinch' attack. Hu97/18, mice compared to Q175FDN mice, had significantly shorter attack latencies. **C.** FVB, Q175FDN, and Hu97/18 mice did not significantly differ on attack frequency. *=difference between indicated bars. *= $p<0.05$. Error bars ±SEM

Compared to FVB mice, of which 66.7% engaged in violent behaviors, 71.4% of Hu97/18 mice and 40% in Q175FDN mice engaged in violent behaviors (Figure 8A). Hu97/18 mice had a similar percentage engaging in violent behaviors in both the NHC (75%) and RI (71.4%) assays despite the fact that the RI test is considered more threatening. This indicates that the Hu97/18 mice reacted similar to both the NHC and RI assay. Additionally, the reduction of Q175FDN mice participating in violent behavior is consistent with the results found in the NHC assay where 0% engaged in violent behavior, which is less than the 14% of FVB mice that did. However, Hu97/18, Q175FDN, and FVB mice did not significantly differ in latency to violent behaviors (Figure 8B, 1 way ANOVA with Turkey's multiple comparisons test compared to

FVB, Hu97/18 p=0.9878, Q175FDN p=0.5494). Based on these data, Hu97/18 mice do not exhibit elevated aggression during the RI assay.

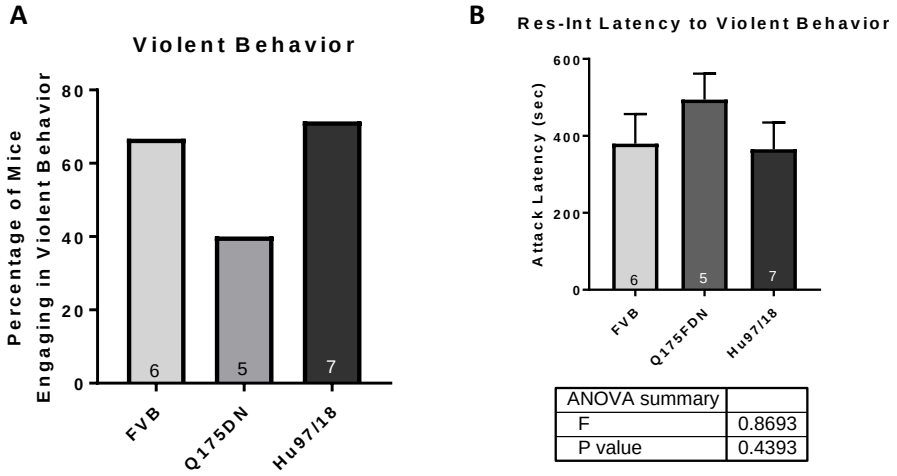


Figure 8: Hu97/18 mice do not exhibited elevated violent behavior in the resident-intruder assay. Mice were tested in RI under red light in the animals dark phase with a max trial duration of 10 min. **A.** Compared to FVB mice, a similar percentage of Hu97/18 mice engaged in violent behaviors and a smaller percentage of Q175FDN mice engaged in violent behaviors. **B.** FVB, Q175FDN, and Hu97/18 mice did not significantly differ on latency to violent behaviors. Error bars ±SEM

CHAPTER FOUR: DISCUSSION AND FUTURE DIRECTIONS

Discussion

As expected the Hu97/18 mice demonstrated higher aggression scores during the NHC task as indicated by the attack time and attack latency scores. Though attack frequency was not significantly different, we did notice that a higher percentage of Hu97/18 mice engaged in violent behaviors that ended the aggression testing, which indicates that Hu97/18 may exhibit a greater number of violent behaviors rather than a greater number of overall aggressive behaviors. However, Hu97/18 did not demonstrate higher aggression scores during the RI task. Violent behaviors were not significantly different either for Hu97/18 mice compared to FVB and Q175FDN mice. However, the Hu97/18 mice appeared to display similar behavior scores in both the NHC and RI tests even though the RI assay is more stressful and should elicit higher aggression. This data indicates that the increased aggressive phenotypes exhibited by Hu97/18 mice may be from finding both aggression tests equally as threatening, as exhibited by equally reactive aggression in both settings. This suggests that HD-related aggression may be based on overreaction.

The focus of the investigation into aggressive behavior will now shift to neurological differences between the aggressive HD mice, Hu97/18, and the non-aggressive HD mice, Q175FDN, as well as comparison of both to control mice. From this analysis, we seek to identify neurological changes that correlate with the aggressive phenotype exhibited by the Hu97/18 mice. This information can be used to identify targets for therapies to reduce HD-related aggressive symptoms in HD patients.

Future Directions

In future studies, we will conduct volumetric analyses of the amygdala and VMH to see if the volumes of these structures are correlated to the aggressive behaviors exhibited by HD mice. Currently, brains have been collected and are being sectioned and stained. We expect that mice demonstrating higher aggression will exhibit changes in volume of the VMH and amygdala.

Overall, we have found that our humanized HD model exhibits a quantifiable increased aggressive phenotype compared to wild-type controls and our knock-in mouse HD model mice. This aggressive phenotype appears to indicate that HD related aggression is an overreaction to threatening stimuli, which has furthered our understanding of HD-related aggression as a reactive form of aggression. Using our humanized HD model, which recapitulates HD-related aggression, we can elucidate the neurological changes that can correlate to this heightened aggressive phenotype and identify neurological targets for therapeutics to reduce aggressive behavioral symptoms in HD patients.

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