Phenotyping the ΔIg3-MuSK Mouse Model Using Automated Continuous Behavior Monitoring

By
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Thesis

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Abstract

The MuSK-BMP signaling pathway has been implicated in neuromotor and cognitive regulation, and adult hippocampal neurogenesis, yet its behavioral consequences remain insufficiently understood. To selectively investigate the behavioral consequences of disrupting MuSK-BMP signaling in vivo, the current study employs Automated Continuous Behavior Monitoring (ACBM) to characterize the ΔIg3-MuSK mouse model, which lacks the Ig3 domain required for BMP co-receptor function. ACBM is a computer vision-based machine learning model that enables high-resolution, sensitive, and unbiased quantification of nine defined behaviors across five days in individually housed mice. ΔIg3-MuSK and wild-type mice of both sexes were recorded at five months of age and their behavior was analyzed across zeitgeber time.

Our findings revealed previously undetected, time of darkness-specificity and sexually dimorphic behavioral phenotypes associated with MuSK-BMP signaling disruption. The ΔIg3-MuSK males exhibited early dark hypoactivity, characterized by reductions in neuromotor (walk, rear) and ingestion (drink, eat-by-hand) behaviors. ΔIg3-MuSK females displayed a similar pattern of reduced neuromotor (walk) and exploratory (sniff) behaviors during early dark hours. However, the ΔIg3-MuSK females showed striking spikes in neuromotor (hang) and ingestion (drink, eat) behaviors during late dark hours, which were absent in males. Other sexually dimorphic behaviors observed were sustained elevation in groom in males and reduction in sniff in females throughout the entire dark period. Comparative analysis with 5xFAD, an Alzheimer's disease mouse model, highlights divergent patterns: whereas ΔIg3-MuSK demonstrated hypoactivity in neuromotor behaviors and sustained elevation in groom, 5xFAD mice showed reciprocal hyperactivity in neuromotor (hang, walk, rear) behaviors and sustained reduction in groom, suggesting model-specific behavioral signatures.

Our data suggest that the loss of MuSK-Ig3-mediated BMP signaling in ΔIg3-MuSK mice may modulate sex-specific neural circuits governing self-care, foraging, and stress-related

behaviors. These findings establish the power of ACBM for detecting subtle, temporally defined, and long-term behavioral phenotypes in the ΔIg3-MuSK model, with implications for understanding neuromotor function, mood regulation, and neurodegenerative disease mechanisms by providing sensitive and reproducible behavioral readouts.

Chapter 1: Introduction

I. Muscle Specific Kinase (MuSK) and its Role in Neuromuscular Junctions

The neuromuscular junction (NMJ) is a highly specialized cholinergic synapse critical for fast, fatigue-resistant, and precise communication between motor neurons and muscle fibers, playing an important role in motor coordination, muscle strength, and overall neuromotor function (Hall & Sanes, 1993; Sanes & Lichtman, 1999). At the NMJ, acetylcholine is released from presynaptic motor neurons, activating acetylcholine receptors clustered on the postsynaptic muscle membrane, thereby initiating muscle contraction. The stability of this synapse is orchestrated by multiple signaling pathways, of which muscle-specific kinase (MuSK) is a central component (Fish & Fallon, 2020).

MuSK is a transmembrane receptor tyrosine kinase that consists of an ectodomain with three immunoglobulin-like domains Ig1, Ig2, and Ig3, one CRD/Frizzled-like domain, and a cytosolic tyrosine kinase (Fish & Fallon, 2020). MuSK is predominantly localized at the postsynaptic membranes of NMJs and is responsible for the formation, maintenance, and stability of the neuromuscular synapse (DeChiara et al., 1996; Burden et al., 2013). MuSK operates via three different signaling pathways: tyrosine kinase-dependent receptor for agrin-LRP4 signaling essential for NMJ formation (Fig. 1.1A), a receptor for Wnt ligands (Fig. 1.1B), and a Bone Morphogenic Protein (BMP) signaling pathway (Fig. 1.1C), also known as the MuSK-BMP pathway (Fish & Fallon, 2020; Yilmaz et al., 2016).

The Fallon Lab has elucidated the novel MuSK-BMP signaling mechanism where MuSK acts as a BMP co-receptor, independent of its kinase activity, and mediates BMP-dependent transcriptional regulation specifically through its immunoglobulin-like Ig3 domain that confers selective and high-affinity binding to BMP ligands (Yilmaz et al., 2016). The distinct functional domains of MuSK, particularly the MuSK-Ig3 domain mediating BMP signaling, suggest a functional modularity critical for specific cellular responses. This positions the MuSK-BMP pathway, and specifically the Ig3 domain, as a promising therapeutic target, offering selective

modulation of BMP signaling without the systemic side effects associated with broader BMP inhibition (Manzari-Tavakoli et al., 2022).

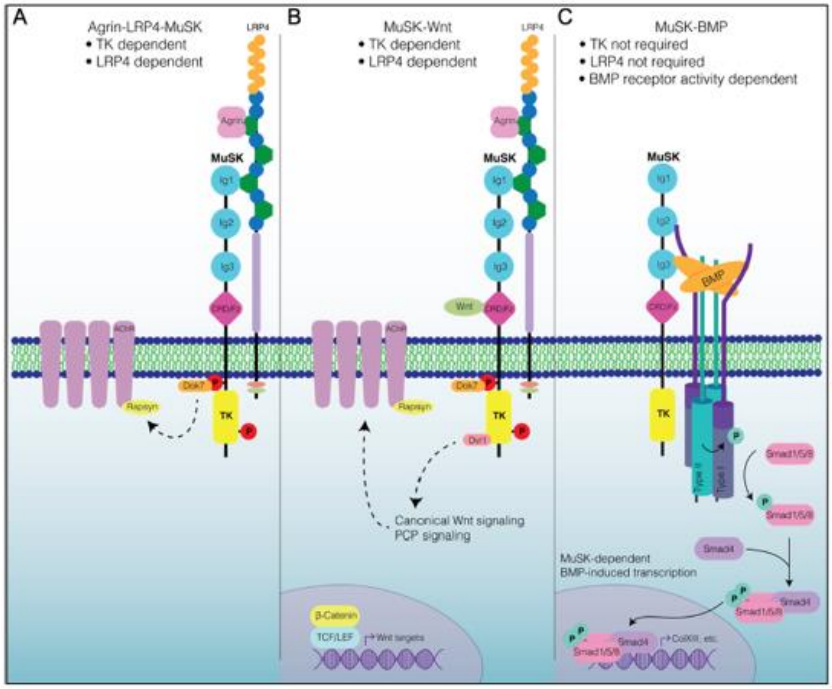


Fig 1.1 MuSK structure and signaling pathways *(A) Canonical agrin-LRP4-MuSK signaling stabilizes AChRs at the postsynaptic membrane. (B) MuSK-Wnt signaling. (C) MuSK-BMP signaling mediated via the MuSK-Ig3 domain. Note the requirement of intracellular BMP receptor machinery in contrast to MuSK tyrosine kinase requirement for agrin-LRP4 signaling. Source: figure and legend adapted from Fish & Fallon 2020*

II. <u>ΔIg3-MuSK mouse model</u>

To investigate the specific functions of the MuSK-BMP pathway in vivo, the Fallon Lab generated the ΔIg3-MuSK mouse model that constitutively expresses MuSK lacking the Ig3 domain, the binding site for BMP. The ΔIg3-MuSK mouse model was genetically engineered using CRISPR-Cas9 to delete the MuSK Ig3 domain-encoding exons 6 and 7 (Fig. 1.2). The genetic deletion selectively abolishes MuSK-mediated BMP signaling, while preserving the traditional agrin-LRP4-dependent MuSK signaling functions mediated by Ig1 to isolate the functional significance of BMP signaling from other pathways mediated by MuSK (Jaime et al., 2024; Yilmaz et al., 2016).

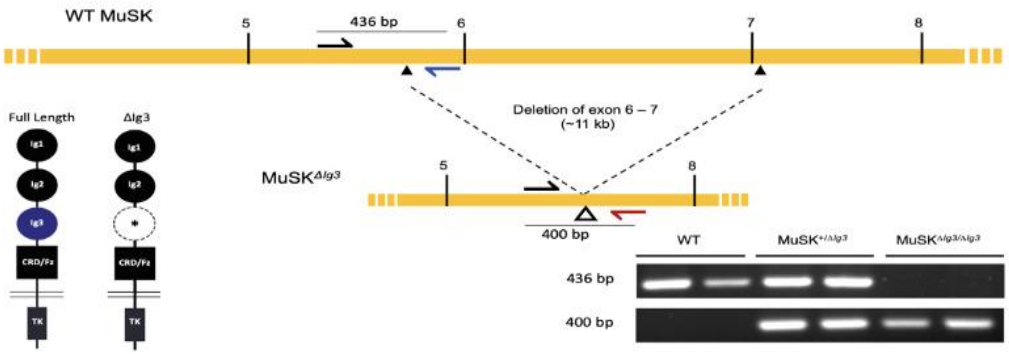


Fig 1.2 ΔIg3-MuSK mouse model *Schematic of the ΔIg3-MuSK mouse model using CRISPR-Cas9 to delete the MuSK-Ig3 encoding exons 6 and 7. The bottom right is the PCR amplification of WT and ΔIg3-MuSK allele in WT, ΔIg3-MuSK heterozygous, and ΔIg3-MuSK homozygous mice using allele specific primers. Source: figure and legend adapted from Jaime et al., 2024.*

Homozygous ΔIg3-MuSK mice are viable and fertile showing normal levels of NMJ innervation and stemness, with cultured myogenic ΔIg3-MuSK cells showing significantly reduced BMP4 signaling (Jaime et al., 2025; Yilmaz et al., 2016). Collectively, these observations emphasize the unique molecular and functional role played by MuSK's Ig3 domain and underscore ΔIg3-MuSK mouse as a valuable tool for understanding the MuSK-BMP signaling pathway's mechanistic and therapeutic potential in various neuromotor (Jaime et al. 2025) and cognitive contexts (Xi, 2024).

III. Significance of the MuSK-BMP Pathway

MuSK's interaction with BMPs modulates cellular processes such as proliferation, differentiation, and quiescence. For instance, the pathway plays a role in muscle stem cell quiescence and myofiber size regulation, thereby adding complexity to MuSK's role in neuromotor physiology and muscle biology (Madigan et al., 2024). ΔIg3-MuSK have been shown to exhibit selective neuromuscular features, where three-month-old ΔIg3-MuSK mice show smaller myofibers specifically in slow muscles (e.g. the soleus) due to disrupted Akt-mTOR signaling pathways that are crucial for protein synthesis and maintenance of muscle fiber size. This selective muscular vulnerability emphasizes MuSK-BMP signaling's muscle-type specificity

and potential role in therapeutic targeting to combat muscle atrophy (Jaime et al., 2024). Other studies have revealed that the MuSK-BMP pathway also influences muscle growth and repair by regulating muscle stem cell quiescence and activation. At five months, the fast-twitch muscle tibialis anterior of ΔIg3-MuSK mice exhibited reduced muscle stem cell density, accompanied by elevated myofiber size and neuromotor functions demonstrated by an increase in muscle weight and grip strength (Madigan et al., 2024). These demonstrate the distinct and stage-specific contributions of MuSK's Ig3 domain in slow and fast muscle regulation and maintenance across the lifespan.

MuSK is sparsely expressed in the brain and one of the cells that MuSK is expressed in is in the neural stem cell (NSC). Research from the Fallon lab has illuminated the broader role of the MuSK-BMP signaling beyond neuromuscular contexts, revealing its influence on adult hippocampal neurogenesis (AHN), which is the process of generation of new neurons from adult NSCs in the hippocampus (Toda et al., 2019). Many studies support the presence of AHN in rodents, specifically in two neurogenic niches: the subventricular zone of the lateral ventricle (Doetsch et al., 1999) and the subgranular zone of the dentate gyrus (Altman & Das, 1965; Ribeiro & Xapelli, 2021). AHN is also a crucial process in cognitive functions and mood regulation in humans (Kempermann et al. 2018; Hill et al. 2015). The Fallon Lab has demonstrated that ΔIg3-MuSK mice have enhanced AHN and improved hippocampal-dependent cognition (Fallon Lab, 2024). BMPs have been suggested to act as negative signals that inhibit both the activation of NSCs and the integration of immature neurons. Increased BMP signaling has been found in various neuropsychiatric disorders, including Alzheimer's Disease (AD) and major depressive disorder, as well as normal aging. This dysregulation of BMP signaling has been associated with the disruption of AHN (Toda et al., 2019; Fallon Lab, unpublished findings; Mu & Gage, 2011; Babcock et al., 2021; Mira et al. 2010; Tunc-Ozcan et al., 2021).

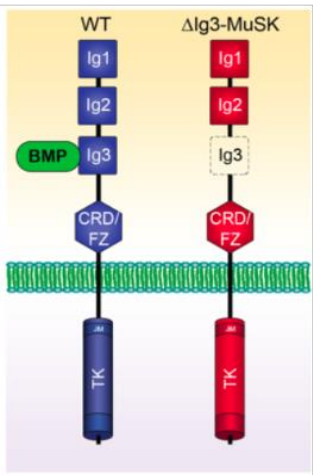


Fig. 1.3 Schematic structure of WT and ΔIg3-MuSK *The extracellular full-length isoform (left) contains three immunoglobulin-like domains (Ig1, Ig2, Ig3) as well a CRD/Fz and an intracellular tyrosine kinase domain. ΔIg3-MuSK lacks the BMP-binding Ig3 domain, leading to disrupted BMP signaling. Source: figure and legend adapted from Jaime et al., 2024*

IV. Automated Continuous Behavior Monitoring (ACBM)

Behavioral phenotyping of mouse models plays a critical role in advancing the understanding of mechanisms and treatments for neuromuscular and neurological diseases. Accurate and sensitive behavioral phenotyping can identify subtle but significant alterations in neuromotor and cognitive functions, which are critical for linking molecular perturbations to functional purposes like detecting disease onset, progression, and response to therapeutic interventions. Traditional behavioral assessments often rely on episodic, observer-dependent tests, such as open field tests and novel object location, that have inherent limitations including short-duration observations, susceptibility to human bias and error, and reproducibility issues across different studies. There is a need for robust, objective, and continuous phenotyping approaches that can reliably detect progressive and subtle behavioral changes, especially in age-dependent and slowly developing phenotypes typical of neurodegenerative diseases.

ACBM is a computer-vision-based machine learning model that enables unbiased, long-term, high-resolution mice behavioral monitoring of freely behaving mice in the home-cage environment with minimal human interference (Jhuang et al., 2010). Specifically, each session involves the recording of individually housed mice for a period of five days, yielding

approximately $1.3x10^7$ frames per mouse per session. ACBM combines motion and position-velocity-based features with a classifier to automatically identify and quantify specific mouse behaviors, creating a behavioral profile for mouse models across 24 hours, with the ability to detect behavioral differences between every hour of recording. The differences in duration spent engaging in each behavior can be compared across multiple dimensions including genotype, sex, and age.

The enhanced sensitivity and reproducibility of ACBM enable more precise characterization of disease-specific phenotypic patterns and longitudinal behavioral changes. Previous studies have successfully applied ACBM to characterize mouse models of neurodegenerative diseases, including AD using 5xFAD (VonEckartsberg., 2024) and amyotrophic lateral sclerosis-frontotemporal dementia using TDP-$43^{Q331K/Q331K}$ mouse models (White et al., 2018). In these contexts, ACBM revealed distinct behavioral phenotypes significantly earlier than traditional observational methods, such as early activity onset and novel grooming profile in 5xFAD, and attentional deficits along with phenotypic heterogeneity in cognitive performance in TDP-$43^{Q331K/Q331K}$ mice. The ability to detect these early, disease-specific behavioral changes highlights ACBM's potential to facilitate timely interventions, more accurate and standardized modeling of disease progression, and can potentially be used for examining treatment clinical efficacy for therapeutic drugs and interventions.

V. Objectives of the Current Study

Despite significant advances, the precise behavioral and neuromotor consequences of disrupted MuSK-BMP signaling in ΔIg3-MuSK mice remain largely unexplored. Given that the MuSK-Ig3 domain is important for the regulation of BMP signaling and the pathway's involvement in the regulation of NSCs and muscle stem cells, the current study aims to characterize the behavioral phenotypes of ΔIg3-MuSK mouse model using ACBM. Specifically trying to understand: 1) What are the behavioral phenotypes present in ΔIg3-MuSK mice? 2) Are

these phenotypes different between males and females? 3) How are ΔIg3-MuSK phenotypes different from other mouse models of AD? By systematically phenotyping this mouse model, we will elucidate how the absence of the MuSK-Ig3 domain and MuSK-mediated BMP signaling modulates neuromotor and cognitive functions.

Chapter 2: Materials and Methods

I. Animals

All animal procedures were performed following the regulations set by and approved by the Institutional Animal Care and Use Committee (IACUC) at Brown University.

A total of 18 mice (5 females, 4 males of each genotype) were used in this study, with 9 ΔIg3-MuSK transgenic mice generated as described in Jaime et al., 2022 and 9 age-matched wildtype (WT) mice, which were C57BL/6J ordered from The Jackson Laboratory (JAX). All mice were group-housed in cages of 2-5 mice in the Animal Care facility at Brown University prior to the initial recording session.

II. Weight

Animals were weighed at the beginning and end of each ACBM recording session during both 2 months and 5 months of age (Supp Fig. 4).

III. Recording Session

The ACBM room is located in the animal care facility at Brown University and was kept at a standard 12-hour light/dark cycle running from 8am to 8pm.

Mice were individually housed during the ACBM recording session in separate ACBM cages. To minimize human handling, the mice were housed in the ACBM room for seven days in total during each session, with the initial two days as acclimatization. Recordings were initiated on day three. Animals were single-housed in separate cages after the initial recording session.

Each cage was monitored with a Firefly MV 0.3 MP Mono FireWire 1394a (Micron MT9V022) at 30 frames per second with cameras connected to a workstation with Ubuntu 14.04 using a Firewire card to connect to all cameras (as described in VonEckartsberg, 2024). All 18 mice (9 ΔIg3-MuSK and 9 WT) were recorded at time points of 2 months and 5 months.

IV. ACBM Data Collection

ACBM will record individually housed mice and automated behavioral "calls" are made from these recordings. In particular, the motion, position, velocity, and acceleration-based features are extracted from the video frames of the recordings and fed through a predefined behavioral characterization classifier created from a prior set of training datasets (described in VonEckartsberg., 2024). The semi-supervised machine learning model was trained to identify nine different neuromotor behaviors described as follows:

1. Drink: drinks from a waterspout (ingestion)
2. Eat: reaches for the feeder (ingestion)
3. Eat-by-hand (EBH): uses forepaws to eat while sitting on haunches (ingestion)
4. Hang: hangs from the ceiling bars of the cage (neuromotor)
5. Rear: stands on hind legs with forepaws raised off the ground (neuromotor)
6. Walk: moves using all four legs (neuromotor)
7. Groom: uses forepaws to clean the face or uses one of the legs to clean the body
8. Rest: stays still for an extended period
9. Sniff: sniffs while remaining static

Then, time in seconds per hour spent on different behaviors will be averaged per hour across the five days of recording for each genotype and sex. Ultimately, the system created a behavior profile of different genotypes after categorization of frames and dataset collected from the videos recorded during the session, achieving an overall accuracy of 81% in recognizing and quantifying the 9 neuromotor behaviors, matching the level of agreement seen among human annotators but

with higher efficiency. Currently, our two-month ACBM data is undergoing some technical issues, so the current study focuses on the five-month data.

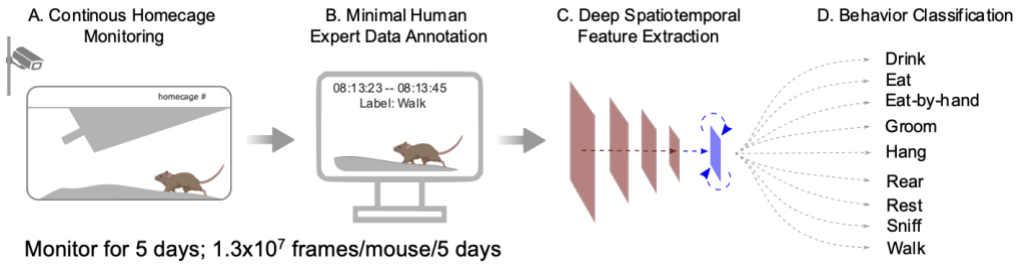


Fig 2.1 ACBM Workflow *Depiction of the workflow from continuous video recording of individually housed mouse to machine learning-based model for feature extraction to the classification of the nine mouse behaviors. Source: figure and legend adapted from Akash Nagaraj in the Serre Lab*

V. Statistical Analysis

Behavioral output from the ACBM pipeline consisted of hourly measurements quantified by average seconds per hour for nine predefined neuromotor behaviors, recorded for each mouse over five consecutive days. The Zeitgeber time (ZT) variable (ZT0-ZT23) and all behavior columns were converted from character to numeric. The full dataset was then stratified by sex to create separate male and female data frames. For each behavior, hourly group summaries (mean ± SEM) were computed by genotype (ΔIg3-MuSK vs. WT). Repeated-measures ANOVA analysis was carried out using a linear mixed-effects model, giving Type III ANOVA tables for the main effects of genotype, hour, and their interaction. We report the main effects of genotype and genotype x ZT interactions. The significance level is set at $\alpha = 0.05$. The criterion for differences in behavioral activities between the genotypes is at least 4 consecutive hours where the error bars of different genotype groups from the repeated measures ANOVA do not overlap.

Chapter 3: Results

I. ACBM reveals differences in behavioral activity between ΔIg3-MuSK and WT males in specific periods of darkness

We analyzed behavioral differences between ΔIg3-MuSK and WT across the entire 24 hours. During the early dark hour period (ZT20-ZT23), ACBM data from five months ΔIg3-MuSK male reveals a reduction in drink, eat-by-hand, rear, and walk relative to WT (Fig. 3.1). ΔIg3-MuSK male also showed reductions in eat and hang, but these were less substantial (Supp Fig. 1). Interestingly, this reduction in behavioral activities seen in the early dark hours was not observed in late dark hours (ZT0-ZT8), with the exception of eat-by-hand. Thus, eat-by-hand was the only behavior that showed a consistent reduction in activity during both early and late dark hours. Across the light hours (ZT9-ZT19), activity profiles for all behaviors, including the low baseline nocturnal activity of mice, were comparable between genotypes. There was no significant difference in rest time during both light and dark hours (Supp Fig. 1).

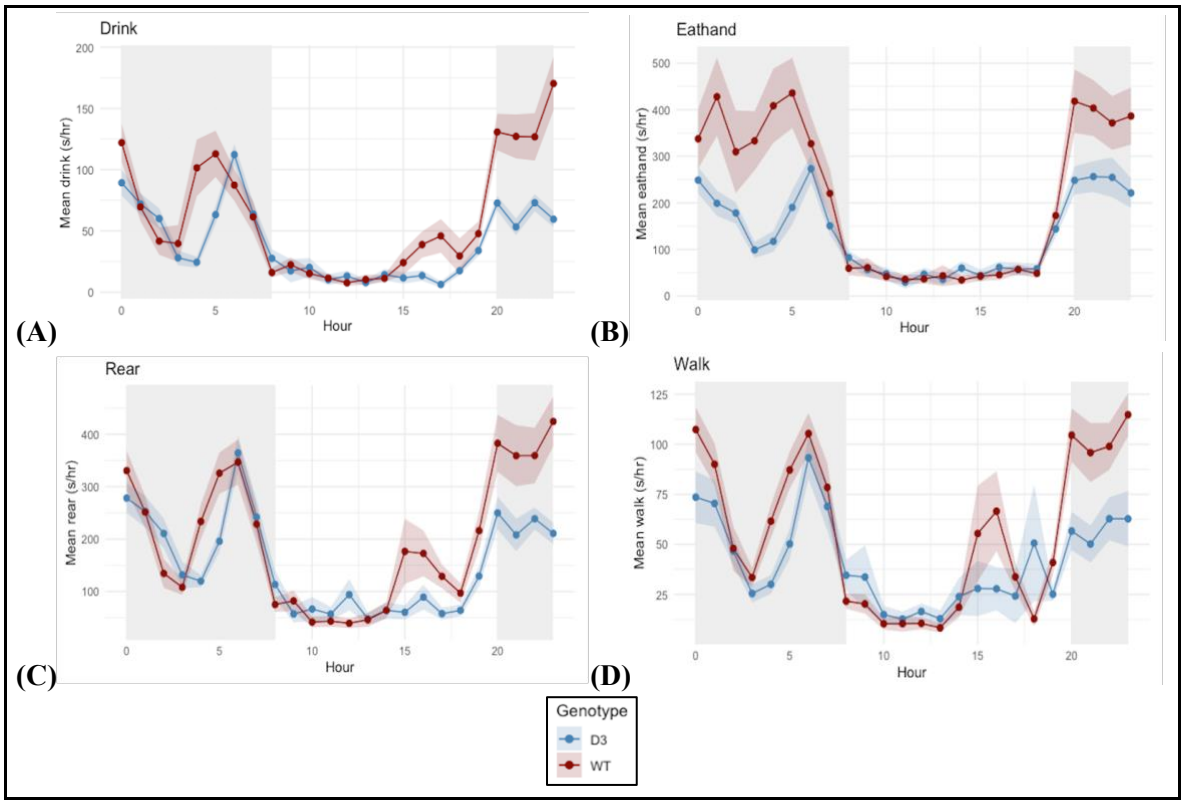


Fig 3.1 ΔIg3-MuSK males show lower Drink, Eat-By-Hand, Rear, and Walk behavior than WT at 5 months during early dark hours *Mean time (s/h) spent (A) drinking, (B) eating-by-hand, (C) rearing, and (D) walking over a 24h time-series cycle in ΔIg3-MuSK (blue) and WT (red) male mice. Lines and shaded bands denote mean ± SEM (n=4 per genotype; see Methods). The light-dark cycle is indicated by alternating backgrounds (white=light hours ZT9-ZT19; gray=dark hours ZT20-ZT8), with early dark (ZT20-23) and late dark (ZT0-8) time periods separated. Behavioral deficits in ΔIg3-MuSK males during the early dark period are indicated by consecutively ≥4h of non-overlapping SEM. ΔIg3-MuSK males exhibit a reduction in Drink, Eat-by-hand, Rear, and Walk throughout early dark (ZT20-ZT23) hours.*

II. <u>ΔIg3-MuSK males display sustained elevation in Groom during dark hours</u>

Interestingly, ΔIg3-MuSK males spent more time grooming than WT throughout both early and late dark hours, peaking at ZT20-ZT21 with mean grooming durations exceeding 1000s/h (Fig. 3.2). No significant groom differences were observed during the light hours.

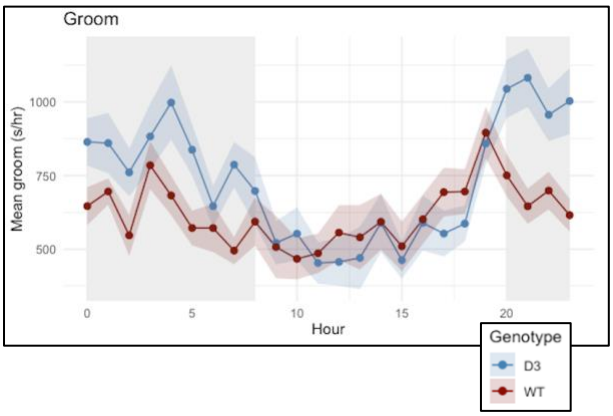


Fig. 3.2 ΔIg3-MuSK males show higher Groom behavior than WT at 5 months during all dark hours *Mean Groom time (s/h) across 24h for ΔIg3-MuSK (blue) and WT (red) male mice. Conventions and cycle shading as in Fig. 3.1. ΔIg3-MuSK males exhibit elevated Groom throughout both early (ZT20-ZT23) and late (ZT1-ZT8) dark hours, peaking at ZT20-21.*

III. <u>ACBM reveals differences in behavioral activity between ΔIg3-MuSK and WT females in specific periods of darkness</u>

We next studied the females. Similarly, in ΔIg3-MuSK females, we observed reductions in walk and sniff behaviors during early dark hours (Fig. 3.3). Interestingly, sniff activity remained lower during all of the dark hours, indicating a persistent sniff deficit. They also showed modest reductions in hang, rear, and eat-by-hand during early dark hours (Supp Fig. 2). There was no significant difference in groom during both light and dark hours between ΔIg3-MuSK and WT females at 5 months. All behaviors during the light period did not differ significantly between genotypes (Supp Fig. 2).

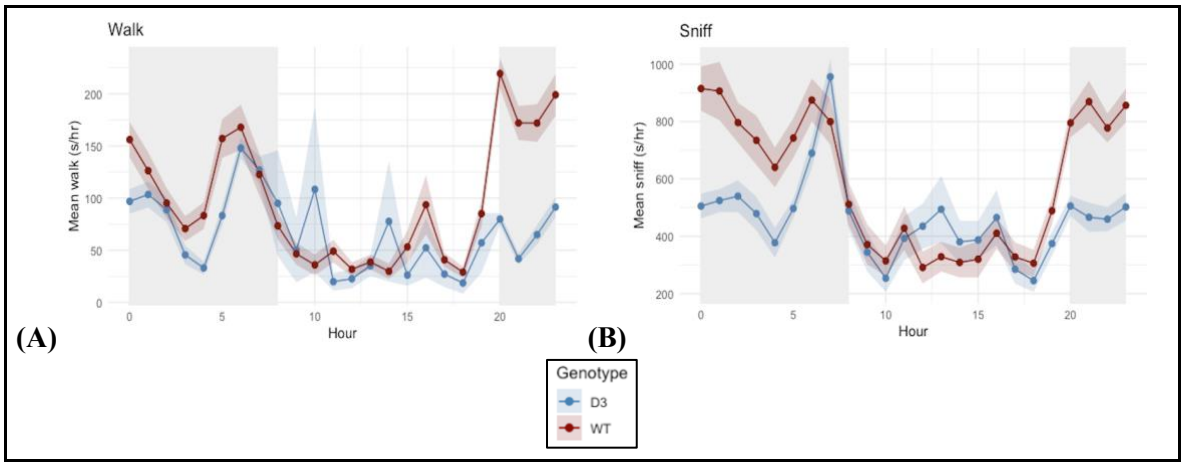


Fig 3.3 ΔIg3-MuSK females show lower Walk and Sniff behavior than WT at 5 months during early dark hours *Mean time (s/h) spent (A) walking and (B) sniffing over a 24h time-series cycle in ΔIg3-MuSK (blue) and WT (red) female mice. Lines and shaded bands denote mean ± SEM (n=5 per genotype; see Methods). Light-dark cycle is indicated by alternating backgrounds (white=light hours ZT9-ZT19; gray=dark hours ZT20-ZT8), with early dark (ZT20-23) and late dark (ZT0-8) time periods separated. Behavioral reductions in ΔIg3-MuSK females compared to WT during the early dark period are indicated by consecutively ≥4h of non-overlapping SEM; ΔIg3-MuSK females exhibit reduced Walk during early dark hours, while Sniff remains suppressed during both early and late dark hours.*

IV. <u>ΔIg3-MuSK females exhibit increased Rest activity during early dark hours</u>

ΔIg3-MuSK females spent more time in rest behavior than WT during early dark hours, while the resting times during the late dark and light hours were comparable between genotypes.

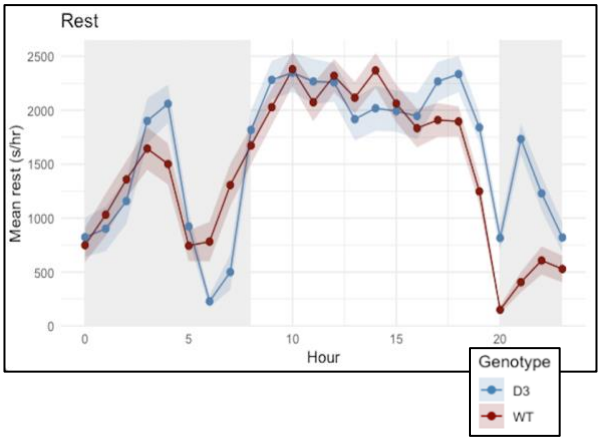


Fig. 3.4 ΔIg3-MuSK females show higher Rest activity than WT at 5 months during early dark hours *Mean resting time (s/h) over 24h in ΔIg3-MuSK (blue) and WT (red) female mice. Conventions and cycle shading as in Fig. 3.3. ΔIg3-MuSK females Rest more than WT during the early dark hours (ZT20-ZT23), with no differences in late dark or light hours.*

V. <u>ΔIg3-MuSK females exhibit spikes in Drink, Eat, and Hang during late dark hours</u>

In contrast to the early dark hours reductions in many behaviors observed in both males and females, ΔIg3-MuSK females exhibit pronounced spikes in drink, eat, and hang during late dark

hours compared to WT. This spiking activity pattern was not found in ΔIg3-MuSK males. For all three of these behaviors, the highest peak of activity occurred around ZT6 (Fig. 3.5). These elevations were limited to late dark hours, and did not persist throughout the remainder of the 24-hour period.

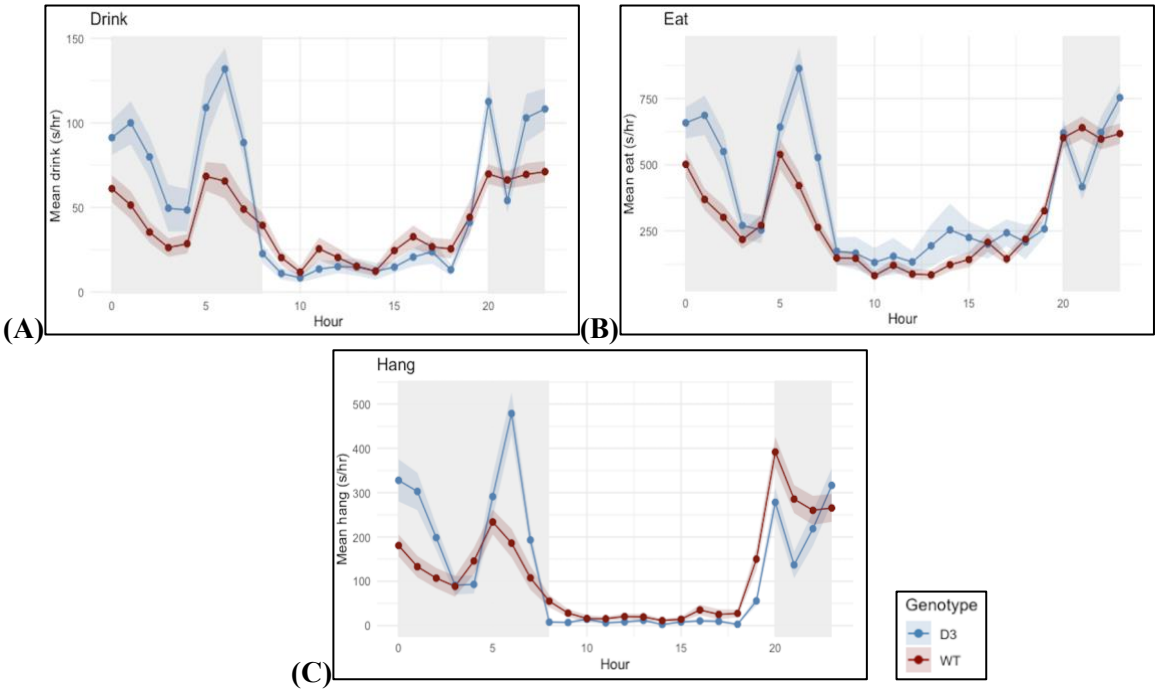


Fig. 3.5 ΔIg3-MuSK females show higher Drink, Eat, and Hang activity than WT at 5 months during late dark hours *Mean time (s/h) spent (A) drinking, (B) eating, and (C) hanging over 24h in ΔIg3-MuSK (blue) and WT (red) female mice. Conventions and cycle shading as in Fig. 3.3. All three behaviors exhibit peaks at ZT6 and consistent elevation during late dark hours (ZT0-ZT8) compared to WT, indicated by consecutively ≥4h of non-overlapping SEM.*

VI. Summary of ΔIg3-MuSK Data

To comprehensively summarize and visualize the period of darkness-specific and sex-dependent behavioral alterations in ΔIg3-MuSK mice, a heatmap table was constructed (Fig. 3.6). Behavioral differences between ΔIg3-MuSK and WT were categorized by sex (male and female) and period of darkness (early dark: ZT 20-23; late dark: ZT 0-8). The directionality of change is denoted by different colors, where red indicates an increase, green indicates a decrease, and white indicates no change.

During early dark hours, male ΔIg3-MuSK mice demonstrated a reduction in ingestion (drink, eat, eat-by-hand) and neuromotor behaviors (hang, walk, rear), along with increased groom behavior. Similarly, females exhibited reductions in neuromotor (hang, walk, rear), ingestion (eat-by-hand), and exploratory (sniff) behaviors. Between the two sexes, four behaviors (eat-by-hand, hang, walk, and rear) overlap in the direction of reduced activity during early dark hours, three of which are all neuromotor behaviors. Out of all nine behaviors, walk showed the most robust and consistent early-dark reduction across both sexes.

Late dark hours showed fewer overall changes, with males showing only three observable phenotypic changes. Male ΔIg3-MuSK mice exhibited a persistent increase in groom and reduction in eat-by-hand throughout the entire dark period. Female ΔIg3-MuSK mice, however, demonstrated striking late-dark increases in ingestive (drink, eat) and neuromotor (hang) behaviors, coupled with a continued reduction in sniff behavior.

From this table, the element of sexual dimorphism becomes apparent, where ΔIg3-MuSK seems to exhibit a female-specific increase in activities drink, eat, and hang during late dark hours. There is a male-specific groom increase and another female-specific reduction in sniff activities.

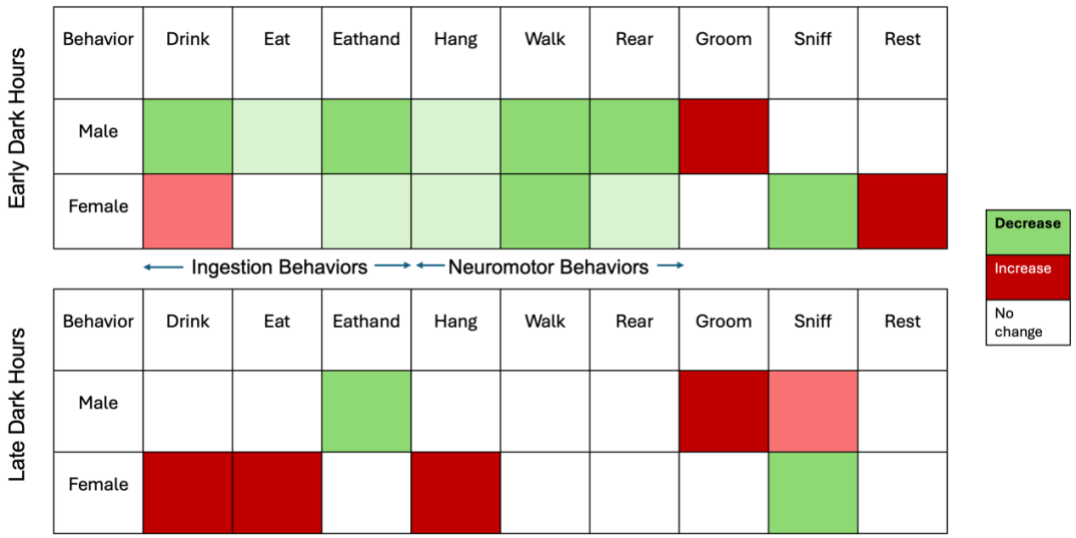


Fig. 3.6 Summary Heatmap for ΔIg3-MuSK compared to WT at 5 months *Display change in behavioral activities of ΔIg3-MuSK males and females during early (top) and late (bottom) dark*

hours. Colors indicate increased (red), decreased (green), or no significant difference (white) relative to WT counterparts. Lighter green and red indicate less substantial changes.

VII. Comparing the ΔIg3-MuSK to the 5xFAD mouse model of AD

Leveraging its high-resolution and quantifiable output database, ACBM allows direct comparison of phenotypes across multiple dimensions, including disease models, age, and sex. To elucidate model-specific behavioral signatures, we compared the behavioral patterns of 5-month ΔIg3-MuSK male mice with those of 8-month 5xFAD male mice (Fig. 3.7). The 5xFAD transgenic mouse model co-expresses five familial Alzheimer's Disease mutations, three human APP and two in human PSEN1, driving rapid amyloid-β plaque deposition beginning at 1.5 months and cognitive decline by 4-6 months of age (Oakley et al., 2006). 5xFAD mice recapitulate key pathological and behavioral hallmarks of AD so they often serve as a benchmark for evaluating intervention strategies and mechanistic pathways.

From our lab's previous ACBM study, 8-months-old 5xFAD males displayed pronounced hyperactivity during the dark period, characterized by increased walking during early and late dark hours, and increased rearing, hanging, and drinking during late dark hours compared to WT controls (VonEckartsberg, 2024). These findings are in contrast with the hypoactivity observed in 5-month ΔIg3-MuSK males. Importantly, hypoactivity observed in ΔIg3-MuSK males occurred during early dark hours, while most elevation in activity found in 5xFAD occurred during late dark hours. Additionally, 5xFAD and ΔIg3-MuSK show the opposite direction of groom activity, where 5xFAD now shows a reduction in groom and ΔIg3-MuSK shows an elevation in groom. Except for sniff, 5xFAD and ΔIg3-MuSK males exhibit opposite phenotypic activity for all behaviors during dark hours. Thus, whereas AD-linked amyloid pathology precipitates hyperlocomotion and reduction in groom, ΔIg3-MuSK yields a reciprocal hypoactive signature and elevation in groom.

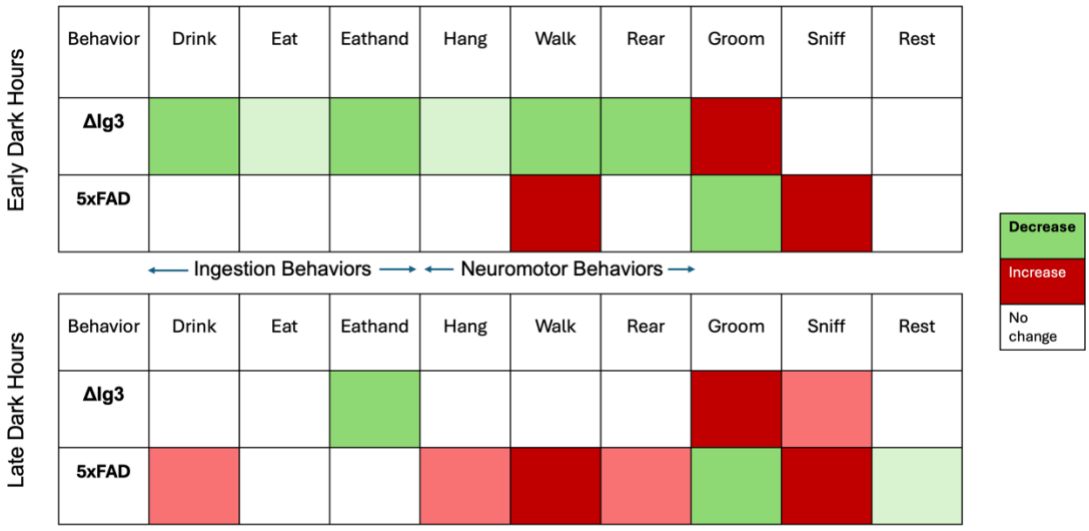


Fig. 3.7 Summary Heatmap for 5 months ΔIg3-MuSK and 8 months 5xFAD males *Display changes in behavioral activities of ΔIg3-MuSK and 5xFAD males during early (top) and late (bottom) dark hours. Colors indicate increased (red), decreased (green), or no significant difference (white) relative to WT counterparts. Lighter green and red indicate less substantial changes.*

Chapter 4: Discussion

I. ACBM reveals period of darkness-specific behavioral differences

ACBM uniquely enables the detection of time-of-day-specific phenotypes by combining long-duration (five-day) video recording with a semi-supervised machine-learning pipeline that classifies nine discrete behaviors in individually housed mice. Unlike traditional assays, which are limited to brief, observer-dependent snapshots, ACBM captures continuous 24-hour activity profiles and can distinguish early (ZT20-ZT23) from late (ZT0-ZT8) dark-period dynamics. In 5-month ΔIg3-MuSK males, ACBM identifies early dark reductions in ingestion behaviors drinking and eating-by-hand, and in neuromotor behaviors walking and rearing (Fig. 3.1). These deficits were absent during the late dark and light hours, highlighting the period of darkness-specificity of the reduction phenotype. In parallel, ΔIg3-MuSK females exhibited early dark hypoactivity in walking, sniffing, eat-by-hand, and rearing (Fig. 3.3, Supp Fig. 2), although the reduction for eat-by-hand and rearing was less pronounced compared to males.

For both sexes, four behaviors, eat-by-hand, hanging, walking, and rearing, consistently trend downwards during the early dark window, three of which (hang, walk, rear) are neuromotor. Walking emerged as the most strongly shared deficit in both males and females, with ΔIg3-MuSK mice spending substantially less time walking throughout early dark hours, while levels during late dark and light hours remained comparable with their WT counterparts. Notably, eat-by-hand remained suppressed across both early and late dark hours in ΔIg3-MuSK males, potentially suggesting a sustained impairment in complex ingestive actions (Fig. 3.6).

This pattern of early dark period hypoactivity in male and female ΔIg3-MuSK may reflect an anxiolytic-like state arising from the disruption of MuSK-Ig3-mediated BMP signaling. Under normal physiology, BMP ligands maintain NSCs in quiescence by signaling through the BMPR-IA, thereby inhibiting AHN (Mira et al., 2010; Mu & Gage, 2011). Loss of Ig3-mediated BMP co-receptor function in ΔIg3-MuSK mice would be expected to release this brake on NSC, enhancing AHN. Notably, inhibition of BMP levels has been shown to produce anxiolytic- and antidepressant-like behavioral profiles through effects on hippocampal newborn neurons (Tunc-Ozcan et al., 2021), and AHN has been demonstrated to be necessary for the behavioral efficacy of chronic antidepressant treatment in mice (Santarelli et al., 2003). BMP signaling has also been implicated in mood regulation and hippocampal neurogenesis (Kim et al., 2021; Mira et al., 2020; Babcock et al., 2021). Together, these findings suggest that disruption of MuSK-Ig3-mediated BMP signaling in ΔIg3-MuSK mice enhances hippocampal neurogenesis and contributes to an anxiolytic-like behavioral phenotype, in contrast to the heightened anxiety and hyperactivity commonly observed in AD patients (Kim et al., 2021). Furthermore, the early dark hour period-specific reduction in walking, a neuromotor behavior highly sensitive to exploratory drive and stress reactivity, supports the presence of an anxiolytic-like signature in ΔIg3-MuSK mice. Future studies combining ACBM with anxiolytic drug administration (e.g. Prozac) in WT mice will be critical to confirm this proposed anxiolytic-like signature in ΔIg3-MuSK mice by establishing the ACBM output for anxiety profiles in WT for comparison.

Although the direct translation of rodent locomotor patterns to human behaviors is complex, by quantifying neuromotor outputs, ACBM has the potential to link targeted molecular perturbation, such as MuSK-Ig3 domain deletion, to the regulation of functional neuromotor circuits in mice.

II. ACBM reveals sexual dimorphisms in behavioral activities

1) ΔIg3-MuSK female-specific late dark hour spikes

During the late dark period (ZT0-ZT8), ΔIg3-MuSK females display increases in drinking, eating-by-hand, and hanging behaviors (Fig. 3.5), a pattern absent in males and indicative of a ΔIg3-MuSK female-specific augmentation of ingestive and neuromotor activities that may be representative of foraging behaviors. Previous studies have demonstrated that female rats take significantly longer to finish foraging than males under both baseline (no-threat) and robot-encounter (threat) conditions, possibly indicating a greater degree of risk-aversion and cautious foraging strategies for female rodents (Meng et al., 2024). Another study showed different post-threat coping strategies between the two sexes, where, unlike males that showed passive defensive freezing, female mice preferentially engage in active behaviors such as foraging and exploration after exposure to a life-threatening stimulus. This may potentially suggest that female mice have an intrinsic drive towards resource-seeking behaviors even after perceived threats (Liu et al., 2022). Together, these observations raise the possibility that the pattern of increased ingestive and neuromotor activities observed in ACBM reflects enhanced and more persistent foraging drive in ΔIg3-MuSK females. This drive could be evolutionarily linked to innate maternal or nursing-related behaviors, where efficient resource acquisition is critical for preparing for pup care and survival, and may become amplified following disruption of MuSK-BMP signaling.

2) ΔIg3-MuSK female-specific sniff reduction in early and late dark hours

ΔIg3-MuSK females exhibit a uniform reduction in sniffing behavior throughout both early and late dark hours, whereas male sniffing remains comparable to WT (Supp Fig. 1). In social approach paradigms, sniff time is a validated index of social motivation, where reduction in sniff behavior is interpreted as social avoidance (Rincon-Cortes, 2023). Here, the persistent decrease in sniffing by ΔIg3-MuSK females may reflect attenuated social motivation or exploratory drive.

3) ΔIg3-MuSK male-specific groom elevation in early and late dark hours

ACBM revealed that ΔIg3-MuSK males consistently groom more than WT counterparts during both early and late dark periods, whereas groom activity was comparable between ΔIg3-MuSK and WT females (Fig. 3.2; Supp Fig. 2). Rodent grooming is an ethologically conserved behavior reflecting both stress-coping mechanisms and maintenance self-care behaviors, which may be analogous to self-grooming in humans (Kalueff et al., 2016). Aberrant grooming has been a core feature of rodent models of neurodegenerative diseases. For instance, the A53T α-synuclein transgenic mice of Parkinson's Disease show impaired self-grooming as early as 1-2 months of age, before overt motor or cognitive deficits emerge (Paumier et al., 2013). Progressive motor ataxia during later stages would also be expected to show a reduction in grooming activity. In contrast, a rat model of the early stages of Huntington's induced by the striatal injection of quinolinic acid leads to aberrant hyper-grooming (Scattoni et al., 2003). These observations suggest that altered grooming behavior may potentially inform early behavioral hallmarks in disease models (Fig. 4.1).

The male- and female-specific behaviors found in ΔIg3-MuSK mice may suggest that MuSK-Ig3-dependent BMP signaling modulates sex-specific neural circuits governing self-care, foraging, and stress-related behaviors. This underscores the importance of incorporating sex as a biological variable in interpreting behavioral phenotypes to improve translational relevance due

to the fundamental differences in neural processes and behavioral responses between males and females (Shansky & Woolley, 2016).

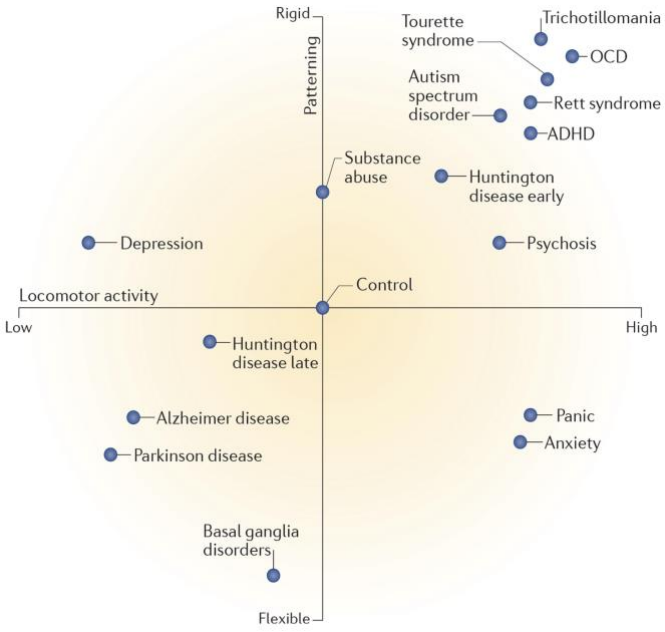


Fig. 4.1 Expected self-grooming behavior in rodent models of neuropsychiatric and neurodegenerative disorders *Aberrant self-grooming is observed in animal models of several neurodegenerative diseases. Shown are the expected rodent self-grooming phenotypes relevant to particular disease models. The x-axis represents the amount of self-grooming activity (from low to high frequency), and the y-axis represents the degree of sequential patterning, ranging from rigid and repetitive to more flexible behavior. The expected behavior of WT control is shown at the center. Note the expected low self-grooming behaviors in AD, PD, depression rodent models. Source: figure and legend adapted from Kalueff et al., 2016*

III. ACBM allows for comparison across different mouse models

The contrasting behavioral patterns between ΔIg3-MuSK and 5xFAD underscore ACBM's utility in resolving, detecting, and comparing nuanced and distinctive behavioral patterns of different mouse models. Disruption of MuSK-BMP signaling through Ig3 deletion in ΔIg3-MuSK males results predominantly in hypoactive phenotypes, whereas AD pathology in 5xFAD induces behavioral hyperactivity (Fig. 3.7), consistent with the observed behaviors of hyperactivity, and heightened anxiety and aggression observed in human AD patients (Kim et al., 2021). The male-specific increase in groom in ΔIg3-MuSK may signify altered affective, stress-coping, or self-

care states unique to ΔIg3-MuSK males, suggesting divergent pathophysiological trajectories in neurodegeneration versus MuSK-BMP disruption. In contrast, self-care or self-grooming behaviors are often impaired in dementia patients due to their memory deficits, loss of fine motor skills, and confusion, consistent with the reduction in grooming observed in the 5xFAD model. These reciprocal behavioral divergences between the two mouse models may implicate MuSK-BMP signaling in the regulation of circuit excitability central to both neuromotor and cognitive domains. One hypothesis is that loss of MuSK-Ig3-dependent BMP signaling may normalize the synaptic overactivity and network dysrhythmias that underlie the hyperactivity in 5xFAD mice. Treating 5xFAD mice with exon-skipping antisense oligonucleotide targeting MuSK-Ig3 or crossing ΔIg3-MuSK and 5xFAD lines will offer a compelling test of this hypothesis– if Ig3 deletion restores AD-driven hyperactivity and ameliorates associated cognitive impairments in AD models, it would establish MuSK-BMP disruption as a modulatory tool on disease-related circuit dysfunction.

IV. Future Directions

To determine the developmental trajectory of MuSK-BMP-driven phenotypes, our lab will extend ACBM monitoring to 8-month-old ΔIg3-MuSK male and female mice alongside age-matched WT controls. This will reveal whether the observed phenotypes in ΔIg3-MuSK mice persist, intensify, or normalize with age, allowing us to distinguish between transient versus progressive behavioral alterations.

Beyond relying on standard error overlap (SEM) analysis and looking at ≥4h of non-overlapping SEM between genotypes, our lab is currently working to implement more robust statistical models to quantify periods of darkness- and behavior-specific differences between genotypes, and account for between- and within-subject correlation.

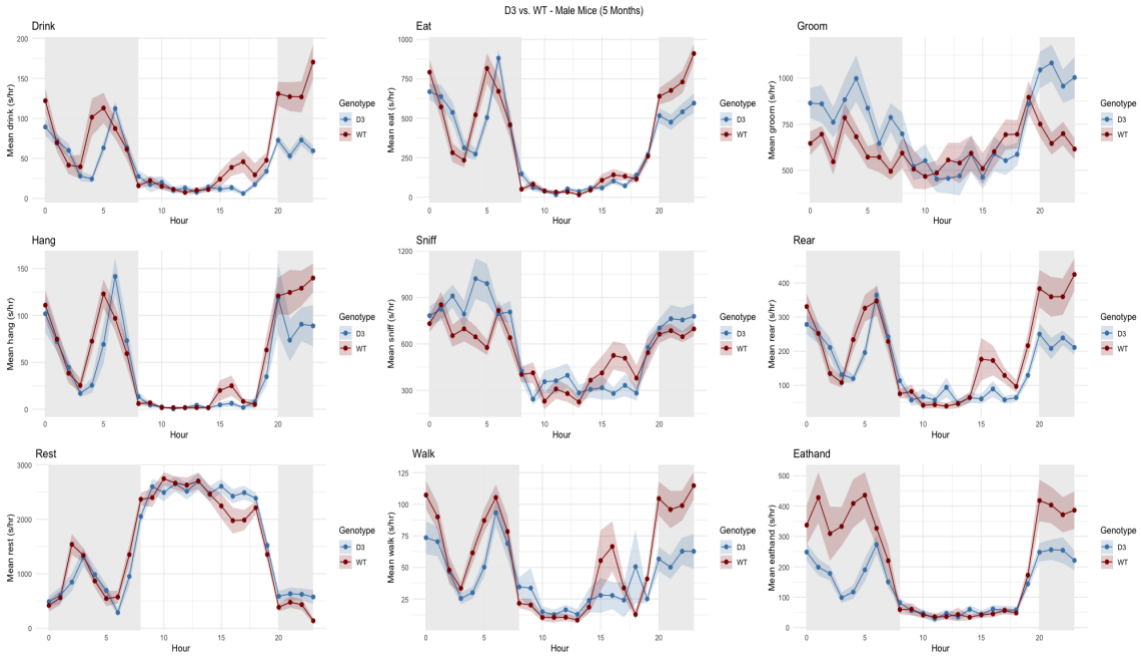
With the current group of n=4 (males) and n=5 (females), statistical power is limited. Expanding each genotype-sex arm to at least n=10 will reduce type II error and strengthen

confidence in periods of darkness-specific and behavior-specific effects. Given the striking sexual dimorphisms observed in the current study, future behavioral studies should emphasize more extensive female sampling to dissect the mechanisms driving this sex-dependent behavioral divergence. Integrating estrous cycle monitoring may help determine how hormonal fluctuations modulate different behavioral phenotypes.

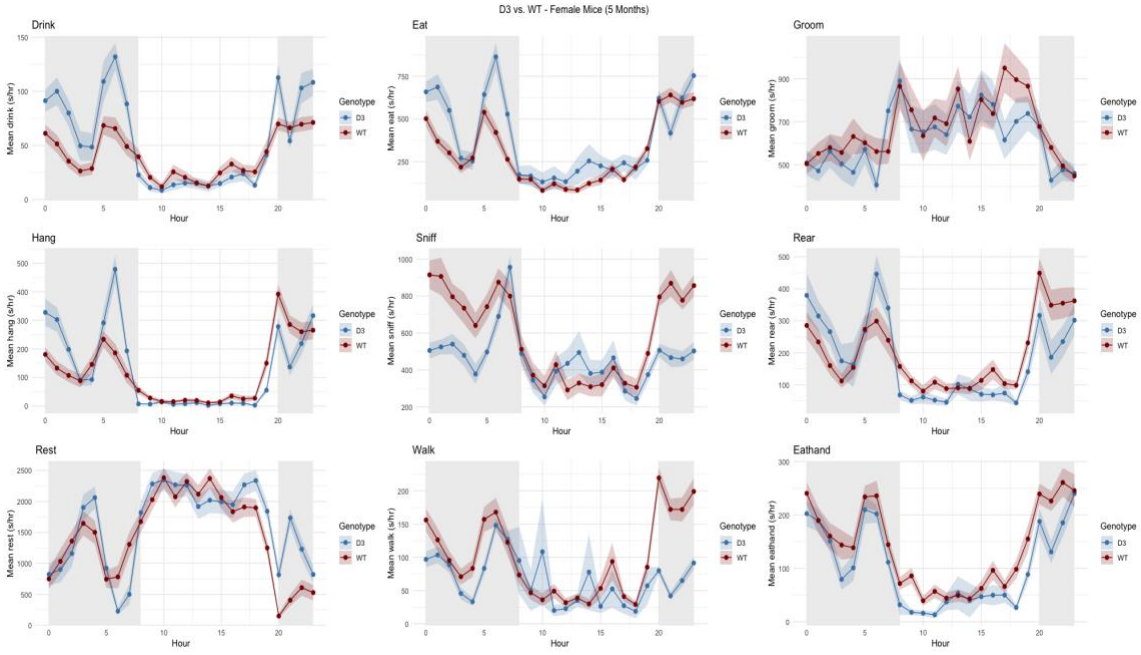
Further refinement of behavioral categorization—grouping the nine recorded behaviors into functional domains (neuromotor, ingestion, exploration, self-care, etc) may reveal correlated phenotypic modules and circuitry insights.

Finally, the large behavioral datasets collected from the automated annotations of ACBM offer a unique opportunity for reproducibility across different testing sites and studies, and standardization for streamlining the ACBM process. Future studies should collate and compare WT recordings from multiple studies to establish baseline WT activity profiles and confirm the reproducibility of ACBM.

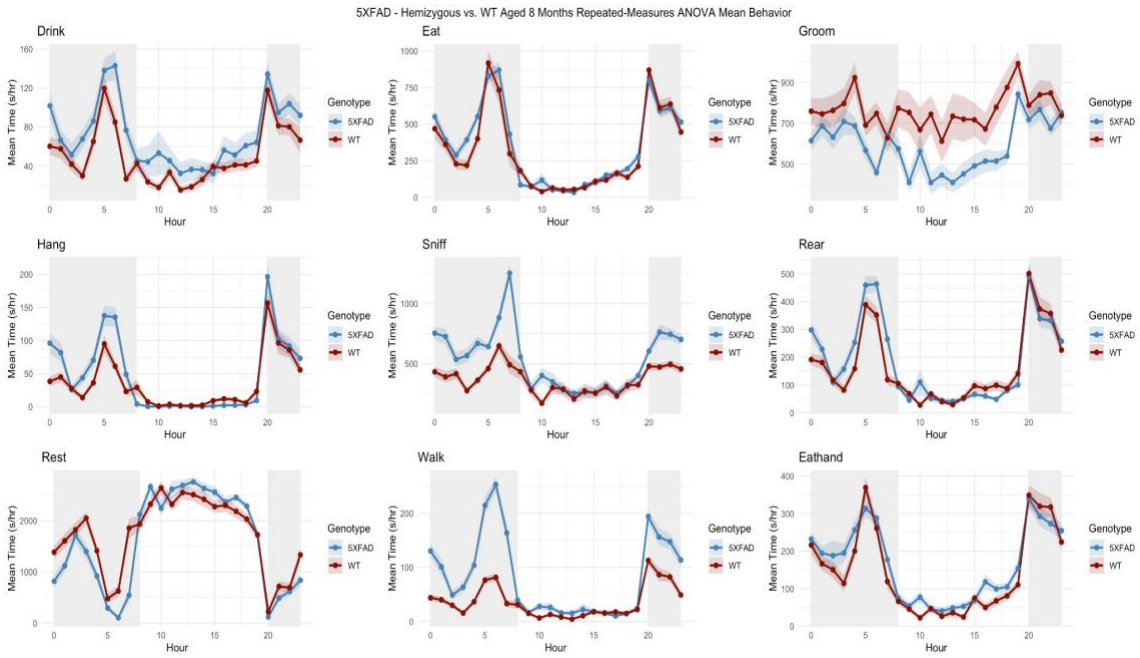
Supplementary Data



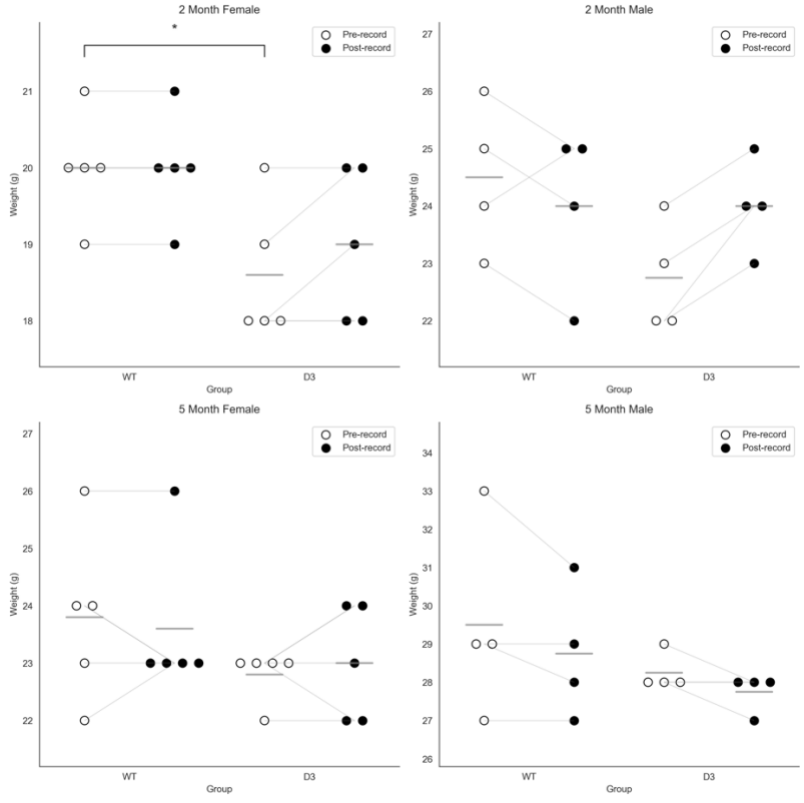
Supp Fig. 1 5-month ΔIg3-MuSK vs. WT Male



Supp Fig. 2 5-month ΔIg3-MuSK vs. WT Female



Supp Fig. 3 5-month ΔIg3-MuSK vs. 8-month 5xFAD Male



Supp Fig. 4 Weight of mice pre- and post-ACBM recording

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