

**PHENOTYPING REGRESSION IN A FEMALE MOUSE MODEL FOR RETT
SYNDROME USING COMPUTATIONAL NEUROETHOLOGY TOOLS**

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Dedication

This dissertation is dedicated to my lovely and supportive wife, Doctor Julia Mykins, and to my two cats (Harley and Odin), dog (Kylie), and two guinea pigs (Peter and Royal), who all quite enjoyed watching me work with videos of mice.

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Abstract

Regression is defined as loss of acquired skills over time and is a key feature of many neurodevelopmental disorders such as Rett syndrome (RTT). RTT is caused by mutations in the X-linked gene Methyl CpG-Binding Protein 2 (MECP2) and is characterized by a period of typical development with subsequent regression of previously acquired motor and speech skills in girls. In human and animal models, it is clear syndromic phenotypes are dynamic over time but phenotyping regression over time in animal models has remained elusive. Lack of established timelines to study the molecular, cellular, and behavioral features of regression in female RTT mouse models is a major contributing factor. Thus, systematic characterization of etiologically relevant behaviors in female *Mecp2*^{heterozygous} (Het) mouse models are needed to move forward. We established an ethologically relevant alloparental pup retrieval task as a model to study cellular mechanisms of complex sensorimotor skills. New deep learning approaches for computational ethology allow for robust analysis of complex behaviors to model endophenotypes and investigate the underlying cellular mechanism in animal pre-clinical models. Using systematic characterization of behavior and histological analysis, we observe adolescent Het have typical perineuronal nets (PNN) in the sensory cortex, mild tactile deficits, and perform efficient pup retrieval. In contrast, adult Het show increased PNN, tactile sensory deficits, and are inefficient at pup retrieval. Using deep learning, we tracked the mice and performed multidimensional analysis on pup retrieval trajectories. Multidimensional analysis revealed two groups of Het: Het that behave like WT, and Het that regress over trials. Surgical degradation of PNNs in the sensory cortex did not rescue behavioral phenotypes in adult Het. We also observe a cell type specific increase in MECP2 expression in adolescent Het, but not adult Het. We speculate the precocious cell type specific increase in MeCP2 expression in adolescent Het may provide compensatory benefits, while the inability to further increase MeCP2 levels leads to regression in adulthood. Thus, we have identified a set of behavioral metrics and the cellular substrates to study regression during a specific

time in the female Het mouse model, which has implications for better designing experimental therapeutics.

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List of Abbreviations, List of Symbols

RTT – Rett syndrome

MECP2 – methyl CpG binding protein 2 human gene

Mecp2 – methyl CpG binding protein 2 mouse gene

MECP2 – methyl CpG binding protein 2, mouse protein

Het – *Mecp2 heterozygous female mouse*

Null – *Mecp2 homozygous male mouse*

WT – wild-type mouse

Het-NR – Het non-regressors

Het-R – Het-regressors

PNN – perineuronal net

PV – Parvalbumin positive GABAergic neurons

S1 – Primary somatosensory cortex

S1BF – Primary somatosensory barrel field cortex

6WO – 6-weeks-old

12WO – 12-weeks-old

w – whisker

b – forepaw

c – whole-body climbing

D0 – postnatal day zero, day of birth of the pups

D# - postnatal day #

DLC – DeepLabCut

NS, ns – not significant, $p > .05$

* - $p < .05$

** - $p < .01$

*** - $p < .001$

**** - $p < .0001$

Dur – duration

Dist – distance

EM – Emax Expected max displacement (Emax)

EMB – Expected max displacement B (EmaxB)

Err – Error

Lat – Latency index

MAcc – mean acceleration

MS – mean speed

MSL – mean step length

Non – nonlinearity

Irr – irregularity

Sin – sinuosity

ST – straightness

PCA – Principal Component Analysis

ChABC – Chondroitinase ABC, an enzyme that cleaves glycosaminoglycan side chains of perineuronal nets

WT-BiPen – WT mouse with bilateral injection of penicillinase in the primary somatosensory barrel field cortex

WT-UniChABC – WT mouse with unilateral control injection of penicillinase in the left primary somatosensory barrel field cortex and unilateral injection of ChABC in the right primary somatosensory barrel field cortex

Het-BiPen – Het mouse with bilateral injection of penicillinase in the primary somatosensory barrel field cortex

Het-BiChABC Het mouse with bilateral injection of ChABC in the primary somatosensory barrel field cortex

Het-UniChABC – Het mouse with unilateral control injection of penicillinase in the left primary somatosensory barrel field cortex and unilateral injection of ChABC in the right primary somatosensory barrel field cortex

PKS – peaks in velocity, also referred to as sub-movements in some literature

TMV – time to max velocity

MSJ – mean squared jerk

CHAPTER 1: INTRODUCTION

The impact of Rett syndrome on society

Rett syndrome is a monogenic neuropsychiatric disorder which primarily affects 1:10,000 females. The exact number of Rett patients is not known, however, it is estimated there are 19,000 patients in the United States alone. Patients require 24/7 care due to clinical symptoms related to neurological, gastrointestinal, cardiac, endocrine, and orthopedic deficits. Despite these clinical manifestations, patients can live well into their 50's with supportive and palliative care. On average, families spend \$40,326 per year on healthcare costs that cover home/hospice visits, therapeutic services, and in/outpatient visits (May et al., 2023). With an estimated 19,000 patients, healthcare resource utilization and costs result in an estimated loss of \$766,194,000 per year in the U.S economy. Rett Syndrome also places a psychological and emotional burden on families due to the impact of patient care on quality of life and the loss of potential productivity for affected individuals (Killian et al., 2016; Laurvick et al., 2006). The magnitude of this societal burden is significant and supports the need for future therapeutic development. Despite two decades of research there are no therapeutic targets which improve the quality of life for Rett patients or their care givers. Given the financial and societal burden of Rett syndrome, there is an exponential need to develop effective therapies for curing or restoring daily function to patients.

Onset of symptoms in female Rett patients

Female patients experience a variety of health problems within four major areas: Neurological, motor, gastrointestinal and growth and development. Patients have a typical development until 6 months where they begin to show signs of developmental regression. If infants have acquired skills to sit up, maintain posture, crawl or walk, they lose these skills. The onset of regression is preceded by repetitive hand movements such as wringing and clapping, and loss of purposeful hand movements such as grasping **Figure 1.1**. These motor phenotypes evolve into tremors and parkinsonian phenotypes over time. The appearance of these motor deficits concurs with

gastrointestinal issues and bone, and growth development issues as highlighted in **Figure 1.1**. Individuals with Rett Syndrome later develop complex cognitive and behavioral dysfunction, including problems with attention, anxiety, aggression, and irritability. Despite these cumulative deficits, patients can live well into adulthood but require 24/7-hour caregivers who are often family members or hospice workers. Amongst caregivers, one of the highest concerns is to restore hand use and social communication to patients (Neul et al., 2023). Currently, there are no FDA approved therapeutics which restore function to social and motor skills in patients, highlighting the need to develop meaningful therapies aimed at preventing regression or restoring sensory and motor function to patients.

One approach is to better design therapies to identify characterize the ‘typical’ period of development which precedes the onset of motor regression phenotypes. Previously it was hypothesized that Rett patients have a ‘typical’ or normal period of development before the onset of regression. However, recent natural history studies of Rett patients have revealed that underlying sensory deficits precede the onset of regression (Buchanan et al., 2019; Einspieler and Marschik, 2019) (**Figure 1.1**). In typical infant development, infants make eye-contact, responsive smiling, pre-linguistic vocalizations (cooing or babbling), and gestures to express their wants and needs. Studies conducted in infants with Rett syndrome observed lack of responsive smiling and frozen smiling (Einspieler et al., 2005; Nomura, 2005; Nomura and Segawa, 1990). Infants with Rett syndrome also show poor response to environmental stimuli such as responding to their name or recognizing familiar adults (Charman et al., 2002; Einspieler et al., 2014; Nomura, 2005).

Collectively, these studies have highlighted that sensory processing and perception issues precede the onset of motor deficits. These underlying sensory deficits in the ‘pre-symptomatic’ stage of patients suggest these deficits are contributing to the higher-order cognitive, social communication and motor deficits observed in the ‘symptomatic’ stage of patients later on. An emerging hypothesis in the field is that Rett Syndrome is a

sensory processing disorder which ultimately gives rise to regression of social communication, motor, and cognition phenotypes over time.

Mutations in MECP2 cause Rett syndrome

In 1966, Andreas Rett documented the first case of Rett syndrome in which he described a female specific pediatric syndrome characterized by brain atrophy (Rett, 1966) (Rett, 1966). Hagberg et al. 1983, 1986 later characterized this as an autism disorder with progressive encephalopathy, loss of motor function, and seizures and hypothesized this was due to an X-linked mutation given its high prevalence in female patients (Hagberg et al., 1986, 1983). In 1999, Amir et al. confirmed this hypothesis when they identified *de novo* mutations in the X-linked gene Methyl-CpG-Binding Protein 2 (MECP2) as the cause for Rett syndrome (Amir et al., 1999). MECP2 is expressed in all cell types and due to random X-inactivation, females are mosaic for functional MeCP2 protein throughout the body. There are now over 600 known mutations in MECP2, and only eight missense and nonsense mutations account for approximately 70% mutations in RTT patients (R106W, R133C, T158M, R168X, R255X, R270X, R294X, and R306C), while large deletions account for 15% of mutations in RTT patients (Ehrhart et al., 2021; Neul et al., 2008; Vashi and Justice, 2019).

Factors contributing to heterogeneity in clinical severity in Rett patient

Significant individual variability in symptom onset and the severity of symptoms is observed in individual female patients throughout their lifetime (Buchanan et al., 2019; Neul et al., 2010). While the exact cellular and molecular mechanism for clinical heterogeneity in patients over time is not known, it is hypothesized this is due to a combination of mutation severity, and X-inactivation skewing. Clinical severity of Rett patients is dependent on the severity of mutations in patients. Hypomorphic mutations such as R133C and R306C are associated with less severe clinical presentation as these mutations still have some protein functionality (Cuddapah et al., 2014; Neul et al., 2008; Vashi and Justice, 2019; Weaving et al., 2003). While amorphic missense mutations such as R106W and T158M, and nonsense mutations such as R168X, R255X, R270X, and R294X cause the most severe phenotypes (Cuddapath et al.,

2014; Neul et al., 2008; Weaving et al., 2003; Vashi & Justice, 2019). Thus, mutation severity is a positive predictor of the degree of symptom severity in patients.

It is also hypothesized that skewed X-chromosome inactivation contributes to the severity of clinical manifestations in Rett patients (Vashi and Justice, 2019). Random X-chromosome inactivation leads to mosaic wild-type MECP2 expression in approximately 50% of most major cell types in the body, including neurons (Samaco et al., 2013; Young and Zoghbi, 2004). While increased X-chromosome inactivation of the functional MECP2 copy is observed in blood samples from Rett patients, the degree of skewing is not correlated with symptom severity (Ishii et al., 2001; Knudsen et al., 2006; Xiol et al., 2019). However, due to limitations on the invasiveness in patients, these studies were conducted on blood sample collections from patients and thus do not address heterogeneity observed in other cell types in the body, such as neurons (Ishii et al., 2001; Knudsen et al., 2006). This is particularly important as there are differences in X-Chromosome inactivation and levels of MeCP2 transcripts between blood and brain cortex samples from patients (Xiol et al., 2019). While these studies suggest X-chromosome inactivation does not contribute to disease severity, given that Rett syndrome is primarily a neuropsychiatric disorder, further work is needed to understand how X-chromosome inactivation contributes to severity of neurological phenotypes. Mouse models for Rett syndrome allow for further investigation of the complex interplay of X-chromosome inactivation, MeCP2 protein expression, and cellular identity contribute to clinical severity in patients.

MECP2 structure and function

MECP2 is a highly disordered 53-kDa DNA binding protein composed of a 486-length amino acid sequence that codes for 6 domains: an N-terminus [NTD; 1-78], Methyl-binding domain [MBD; 78-162], intervening [ID; 162-207], Transcriptional Repression [TRD; 207-310], NCoR interaction [NID; 267-310], and a C-terminus domain [CTD; 310-486] (Ghosh et al., 2010; Good et al., 2021). MECP2 is considered an intrinsically disordered protein (IDP) due to its lack of secondary and tertiary structure (IDP) (Adams et al., 2007; Hite et al., 2012). The majority of typical RTT cases are caused by

missense mutations in the MBD. MECP2s most well-known role is as an epigenetic reader where it binds to methylated DNA via its MBD (Free et al., 2001; Lewis et al., 1992; Meehan et al., 1992). Common mutations such as R106W, R133C, F155S, and T158M mutations result in a loss of MECP2 binding affinity to methylated DNA *in-vitro* (Ballestar et al., 2000). While several studies have demonstrated MECP2 regulates gene expression in an activity dependent manner (Chahrour et al., 2008; Chen et al., 2015; Fuks et al., 2003; Gabel et al., 2015; Jones et al., 1998; Klose et al., 2005; Lyst and Bird, 2015; Tao et al., 2009; Zhou et al., 2006), the exact role of MECP2 is still debated within the field (Tillotson and Bird, 2020).

MECP2 is hypothesized to function as a master gene regulator with diverse functions throughout the cell or to primarily function as an epigenetic repressor of gene expression through its interactions with the nuclear receptor co-repressor complex (NCoR complex) (Lyst & Bird, 2015; Tillotson & Bird, 2020). Mutations within TRD, which binds NCoR, result in RTT phenotypes such as learning and memory deficits and motor dysfunction (Ebert et al., 2013; Heckman et al., 2014). Activity dependent phosphorylation of Threonine 308 (T308) in the TRD domain of MECP2 regulates interactions with NCoR (Ebert et al., 2013). Knock in mutations in mice bearing the common human RTT missense mutation R306C, prevent T308 phosphorylation (Ebert et al., 2013). Loss of T308 phosphorylation decreases repression of gene expression in neuronal culture and coincides with RTT like phenotypes in mice, suggesting that the loss of T308 phosphorylation might contribute to RTT (Ebert et al., 2013).

Through this mechanism it is hypothesized that NCoR binds to MECP2 bound heterochromatin and forms liquid condensates that allow for global gene repression. Mutations in the NCoR binding domain result in significant reduction in MECP2 heterochromatin condensates and increased gene expression in Het mouse brain slices (Li et al., 2020). These findings combined could support that mutations in the TRD domain of MECP2 affect its ability to repress gene expression through NCoR ultimately leading to RTT like phenotypes. However, the present study did not perform behavioral studies to complement these findings. More work in female mouse models of Rett

syndrome is needed to understand the exact mechanism of how perturbing MECP2 ability to bind to NCOR ultimately leads to sensory and motor impairments in females and women with Rett Syndrome. Systematic characterization of behavior over time is critical for understanding how mutations ultimately give rise to complex behavioral phenotypes.

MECP2 is required for neuronal function in the adult Brain

MECP2 is expressed throughout all cell types in the body. Although MECP2 is ubiquitously expressed, it is the most critical for proper neuronal function. MECP2 is expressed at near histone levels (Skene et al., 2010) in neurons and conditional germline deletion of MECP2 in the brain of mice recapitulates RTT like phenotypes (Chen et al., 2001; Guy et al., 2001). MECP2 expression increases postnatally in wild-type rodent cortex, concomitant with the emergence of appropriate critical periods, and is thought to be stable in adolescence and adulthood (Braunschweig et al., 2004; Skene et al., 2010). It was thus previously hypothesized that loss of MECP2 in early postnatal development leads to improper neuronal development and function. However, we now know that MECP2 is essential for adult neuronal function and maintenance. Genetic re-expression of MECP2 in symptomatic male mice that lack *Mecp2* rescues neurological phenotypes, and conditional deletion of *Mecp2* in adult WT male brain recapitulates neurological phenotypes (Guy et al., 2007; McGraw et al., 2011). Collectively, this findings support the idea that MECP2 is critical for mature neuronal function in the adult brain.

Despite this observation, it is still unclear if MECP2 expression is stable over age, and if wild-type MECP2 expression in Het is similar to the age-matched controls (WT). Braunschweig et al, previously showed that wild-type MECP2 expression is decreased in Het; however, the brain region and age were not reported (Braunschweig et al., 2004). MECP2 is highly expressed in GABAergic interneurons over excitatory pyramidal neurons within the same region (Ito-Ishida et al., 2015; Takei et al., 2021). Due to MECP2 significant roles in gene regulation (Chahrour et al., 2008; Chen et al., 2015; Fuks et al., 2003; Gabel et al., 2015; Jones et al., 1998; Klose et al., 2005; Lyst & Bird,

2015; Tao et al., 2009; Zhou et al., 2006), and the timing of experience-dependent plasticity (Krishnan et al., 2017, 2015; Billy Y.B. Lau et al., 2020; Morello et al., 2018; Noutel et al., 2011; Patrizi et al., 2020; Picard and Fagiolini, 2019), investigation of MECP2 expression in ethologically relevant contexts in female Het across ages is essential to move forward in unravelling how behavior deficits arise over time in pre-symptomatic adult Het.

Neurological phenotypes in *Mecp2* mutant mouse model for Rett syndrome

Onset of RTT phenotypes in female mouse models for Rett syndrome

Two years after the discovery that mutations in MECP2 cause Rett syndrome, Guy et al., 2001 generated the first *Mecp2* mutant mouse line. This line is now referred to as the B6.129P2(C)-*Mecp2*^{tm1.1Bird/J} (JAX:003890) and is widely used as a mouse model to study Rett syndrome pathogenesis (Guy et al., 2001). Using the Cre LoxP system, they deleted exon 3-4 of the *Mecp2* gene resulting in no functional MECP2 protein in *Mecp2* homozygous males (*Mecp2*^{-/-}) and mosaic expression of MECP2 in *Mecp2* heterozygous females (*Mecp2*^{+/-}). In the dissertation, we will refer to *Mecp2*^{-/-} homozygous males as null males and *Mecp2*^{+/-} heterozygous females as Het females. While both null males and Het female recapitulate neurological phenotypes of RTT patients, null males rapidly deteriorate by 6-8 weeks of age and require humane euthanasia (Guy et al., 2007, 2001). Whereas Het females have a slower onset and can live to 1-2 years of age. Null males are an excellent model to study the cellular function of MECP2, however, the rapid deterioration in health of null males does not allow us to study the role of MECP2 into adulthood. Additionally, female Het are mosaic for MECP2 expression and more appropriately model the Rett patient population.

Around 3 months of age, Het mice begin to show uncoordinated gait and stereotypical hindlimb clasping which progressively worsens to irregular breathing, forepaw clasping and reduced gross motor function. Concurrent with the emergence of motor phenotypes is reduced anxiety and the onset of seizures in late adulthood (**Figure 1.2**). Mouse

models have delayed onset of motor and social communication deficits, as well as the early onset of sensory processing deficits (**Figure 1.2**) similar to those observed in RTT patients. Het are thus an appropriate model to investigate how mutations in MECP2 gives rise to behavior deficits over time.

Tactile sensory processing deficits in mouse models for Rett syndrome

Mice use their whiskers for tactile sensation and navigating their environment. Sensory information received by the whiskers is sent to the primary somatosensory (S1BF) to decode incoming tactile stimuli from the environment. Whisker stimulation activates mechanosensory neurons which project into the principal trigeminal nucleus of the brainstem. Neurons in the brainstem then synapse onto to ventro posteromedial nucleus of the thalamus (VPM) (Meyer et al., 2010). Excitatory cells in the VPM project to the barrel field cortex and relay incoming tactile sensory stimuli. In Layer 4 of the barrel cortex discrete anatomical units, called barrels (given their name due to their unique barrel like structure) are collections of cells that represent each individual whisker on the mouse mystacial pad (Woolsey and Van der Loos, 1970).

In male *Mecp2* null mice, neurons in the barrel cortex have atypical morphology (Lee et al., 2017) and there is reduced transmission in VPM thalamic serotonergic input into the barrel cortex (S1BF) (Lee et al., 2017; Lo et al., 2016; Moroto et al., 2013). When presented with a novel texture during a whisker dependent texture discrimination task, *Mecp2*-null male mice are unable to differentiate between the novel and familiar textures (Orefice et al., 2016). This study found that MECP2 in peripheral somatosensory neuron is critical for tactile sensation in male mice (Orefice et al., 2016). Despite previous experiments which showed that there are physiological deficits in whisker-barrel system of mice, behavioral analysis did not focus on whether there are whisking deficits which would inhibit discrimination of textures. Additionally, it is unclear whether female Het, which are the apt pre-clinical model for Rett syndrome, have whisker specific sensory processing deficits.

Gross motor deficits in female rodent models for Rett syndrome

It is well known that symptomatic *Mecp2* rodent models have gross motor deficits (Guy et al., 2001; Chen et al., 2001). Gait analysis of *Mecp2* null males revealed alterations in stride, coordination, and balance parameters at 4 weeks of age, before the onset of gross motor phenotypes (Gadalla et al., 2014). A study by De Filippis et al used symptomatic 11–12-month-old female *Mecp2* 308 mouse model to study gross motor skills using the single pellet grasping assay. Female mice with this point mutation show less severe phenotypes compared to null male mice, but develop progressive tremors, motor impairments, and hypoactivity in adulthood (Shahbazian, 2002). These mice showed increased errors in reaching and grasping as well as reduced locomotor activity during the dark phase at the end of training (De Filippis et al., 2015). Interestingly, early pre-symptomatic training in gross motor task delays the progression of these motor phenotypes in Het mice (Achilly et al., 2021). While this study showed that there were differences in the execution of gross motor skill at the end of training in symptomatic Het, it is unclear whether the learning or the execution of the fine motor skills is affected in pre-symptomatic Het.

A common assay used to study motor learning and efficiency is called the single pellet grasping assay. In the single pellet grasping assay mice are placed on a restricted diet to increase motivation in reaching for a high reward food pellet (Chen et al., 2014; Whishaw and Pellis, 1990). The mouse begins by lifting its paw, advancing through the gate and pronating its digits to grasp the pellet. Once the pellet is grasped, the paw returns the pellet back to the mouth. This motion is relatively conserved and similar to the motion used by humans during a reaching and grasping task (Klein et al., 2012; Whishaw et al., 1992). Repetitive training results in the emergence of stereotypical motions which increase the chance of successful retrieval, though the mechanism behind this are not fully understood. These findings collectively make it a good model to study the mechanism of fine motor learning and execution. Establishing how wild-type adult mice adapt efficient reaching strategies over time is essential first step for identifying subtle changes in fine motor function in Het.

Regression in female rodent models for Rett syndrome

Regression, defined as a loss or decline in previously acquired skill, is a hallmark of many neurodevelopmental disorders, in particular, Rett syndrome. Despite two decades of research in rodent models for Rett syndrome, it still remains unclear how regression in sensory and motor skills emerges over time. Select studies have shown possible regression phenotypes, for breathing abnormalities in a mouse conditional knockout model (Huang et al., 2016), learned forepaw skill involving seed opening in a female rat model (Veeraragavan et al., 2016), and in skilled motor learning in female mice (Achilly et al., 2021). In these models, deterioration in learned skills and gross motor skills was noted over multiple weeks. However, reliably identifying regression within sensory and social behavioral tasks in apt preclinical models, with specified time resolution, has remained a challenge. This is partly due to previous studies which have focused on null males, which do not live into adulthood. Het female mice have mosaic expression of MECP2, live well into adulthood and thus more accurately model the female patient population. Systematic characterization of etiologically relevant behavior contexts in Het over time combined with histological analysis of neural substrates for plasticity are needed to move forward in understanding the cellular mechanisms of regression, in patients.

Maternal behavior as a model to study Rett syndrome pathogenesis

An ethologically relevant social behavioral assay to study the cellular and neural circuitry basis for social cognition, complex sensorimotor integration, and experience-dependent plasticity is the maternal behavior assay (Dvorkin and Shea, 2022; Krishnan et al., 2017, 2015; Billy Y.B. Lau et al., 2020; Billy Y. B. Lau et al., 2020; Stevenson et al., 2021). In this assay, female nulliparous mice are co-housed with a pregnant female 3-5 days before birth of pups. Once pups are born, we remove pups from the nest and scatter them in the corners of the home cage. Individuals show spontaneous maternal behavior and will retrieve the pups back to the nest, over multiple days (Hemel, 1973; Krishnan et al., 2017; Stevenson et al., 2021; Xie et al., 2023). The contribution of cell type specific neural circuits are well characterized in innate maternal behavior

(Bendesky et al., 2017; Chong et al., 2020; Fang et al., 2018; Kohl et al., 2018; Krishnan et al., 2017; Maynard et al., 2018; Moffitt et al., 2018; Niv et al., 2015; Tasaka et al., 2018; Wu et al., 2014). However, the neural circuit mechanism by which nulliparous female mice adapt repetitive and stereotyped motions over days to efficiently retrieve scattered pups back to the nest is unclear.

This innate task in virgin nulliparous female mice involves multisensory integration of auditory, tactile and olfaction cues to efficiently adapt and retrieve pups (Alsina-Llanes et al., 2015; Beach and Jaynes, 1956; Champagne et al., 2007; Champagne and Curley, 2016; Hemel, 1973; Koch and Ehret, 1989; Lonstein et al., 2015; Stern, 1996, 1990; Stern and Mackinnon, 1978; Xie et al., 2023). This model allows us to neural mechanisms of social interaction and complex sensory processing and how these mechanisms arise in Het over time. Previously we reported adult Het performs poorly at this task compared to WT controls. Het improve like WT in initial days but make more errors and take longer to finish retrieval in later days compared to WT (Krishnan et al., 2017; Stevenson et al., 2021). Conditional knockdown of MECP2 in the auditory cortex of adult female mice impairs pup retrieval, indicating MECP2 in the auditory cortex is important for experience dependent plasticity mechanisms to adapt efficient retrieval over time (Krishnan et al., 2017).

Perineuronal nets as physical substrates for structural plasticity in the brain

Perineuronal nets (PNNs) are specialized extracellular matrix structures which structurally surround the soma and proximal dendrites of parvalbumin positive (PV+) GABAergic interneurons in the cortex (Hartig et al., 1994, 1992; Kosaka and Heizmann, 1989; Nakagawa et al., 1986). PNNs are composed of a collection of over 40 proteins including chondroitin sulfate proteoglycans (CSPGs), heparan sulphate proteoglycans (HSPGs), lecticans, glycoproteins, hyaluronan and many other molecules that are secreted into or interact with extracellular matrix components (Bruckner et al., 2000; Carulli et al., 2006; Fawcett et al., 2019; Fowke et al., 2017; Hartig et al., 1994; Kwok et al., 2010). *Wisteria floribunda agglutinin* (WFA) binds specifically to N-acetyl

galactosamine found on most CSPGs and thus is used as a common immunohistochemical marker to visualize PNNs in the brain (Hartig et al., 1992; Slaker et al., 2016). Exogenous delivery of Chondroitinase ABC (ChABC), an enzyme which specifically cleaves the glycosaminoglycans (GAG) side chains of CSPGs, degrades PNNs as measured by reduction in WFA staining (de Vivo et al., 2013; Lin et al., 2007; Pizzorusso et al., 2002a). Most knowledge on the structure and function of PNNs is known from studies conducted in the visual cortex and hippocampus of rodents. PNNs restrict plasticity as their expression is concomitant with periods of heightened plasticity in the brain and enzymatic degradation of PNNs restores the brain to states of “juvenile” plasticity (Carstens et al., 2021; Carulli et al., 2010; de Vivo et al., 2013; Hou et al., 2017; Miyata and Kitagawa, 2017; Pizzorusso et al., 2002). Exogenous treatment of cortical slices with ChABC increases dendritic spine density (de Vivo et al., 2013). These findings collectively highlight PNNs are important for restricting synaptic plasticity in the brain.

Abnormal PNN expression and altered synaptic plasticity are observed in rodent models for neuropsychiatric disorders. MECP2 regulates timing and expression of perineuronal nets is during periods of heightened plasticity (Carstens et al., 2021, 2016; Donato et al., 2013; Durand et al., 2012; Gogolla et al., 2006; Hou et al., 2017; Krishnan et al., 2017, 2015; Morello et al., 2018; Patrizi et al., 2020; Pizzorusso et al., 2002a; Sigal et al., 2019). Pup retrieval experience induces an atypical increase in the expression of PNNs in the auditory cortex which coincides with poor pup retrieval performance (Krishnan et al., 2017). Interestingly, ChABC degrades PNNs in the auditory cortex and partially rescues pup retrieval behavior. This suggest other cortical brain regions are important for mediating pup retrieval behavior in adult Het. Using whole brain histological analysis, it was reported that prior to behavior, adult Het have increased PNN expression in the primary somatosensory barrel field cortex (S1BF) (Lau, Layo et al., 2020). In mice, sensory information received by the whiskers is sent to the S1BF to decode incoming tactile stimuli from the environment. The physiological relevance of increased S1BF PNN expression in adult Het is not known. Given the known role for PNNs in inhibitory plasticity, we hypothesize that abnormal increase in PNN expression

in the S1BF restricts incoming tactile sensory stimuli leading to poor performance in tactile relevant task.

As experimental studies move toward using etiologically relevant behavioral assays, there is a shift to studying free moving behavior in natural contexts in order to model the complexity of endophenotypes in neuropsychiatric disorders. We propose that systematic and nuanced characterization of etiologically relevant behaviors in Het over time, combined with histological analysis of neural substrates for plasticity are needed to move forward in understanding the cellular mechanisms of these complex neural phenotypes. Given that Rett syndrome phenotypes are dynamic over time, we hypothesize MECP2 expression is age dependent and coincides with appropriate expression of perineuronal nets required for sensory processing in ethologically relevant contexts.

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Appendix

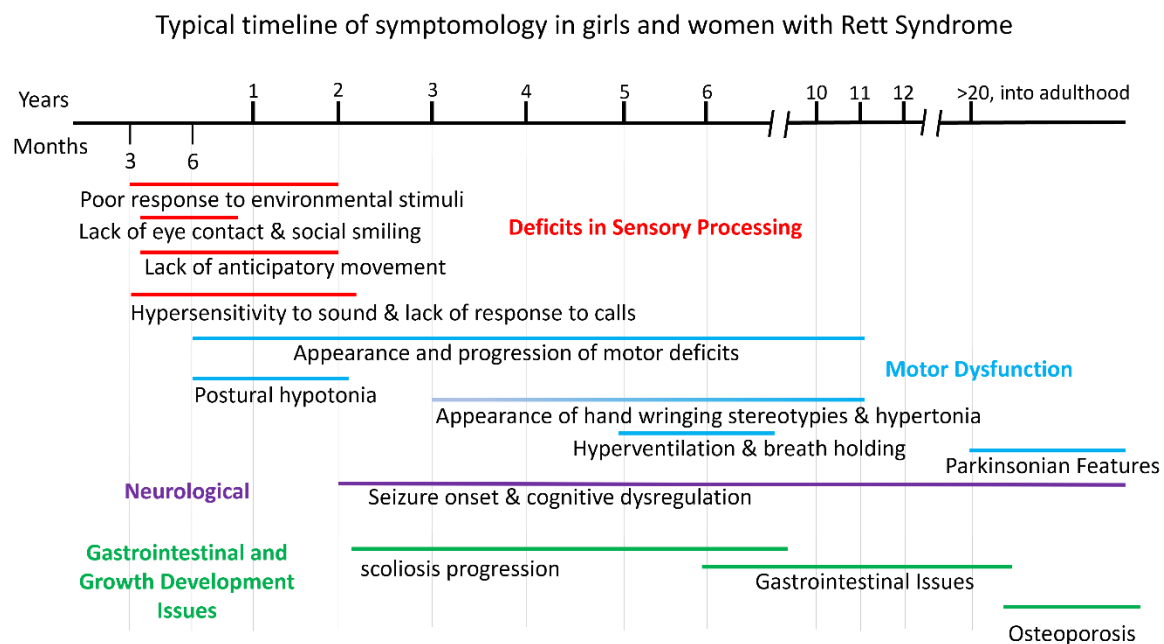


Figure 1.1. Symptomology in girls and women with Rett Syndrome.

Age is indicated at the top in months (bottom) and years (top). Lines indicate the period in which the corresponding stage or symptom appears or has been reported. Red lines indicate the appearance of sensory issues during the 'typical period of development'. Blue lines indicate motor symptom onset. Purple lines indicate other neurological symptoms such as seizures and emotional dysregulation observed in patients. Green lines represent the emergence of peripheral symptoms related to gastrointestinal and growth development issues. Despite these deficits, patients with Rett syndrome can live into late adulthood with 24/7-hour care. This figure is modified from Chahrour and Zoghbi, 2007, and is synthesized with findings collected from Buchanan et al., 2019; Djukic and Valicenti McDermott, 2012; FitzGerald et al., 1990; Jian et al., 2006; Killian et al., 2017; Motil et al., 2012; Neul et al., 2014; Nomura, 2005; Tarquinio et al., 2017, 2018; Vignoli et al., 2010.

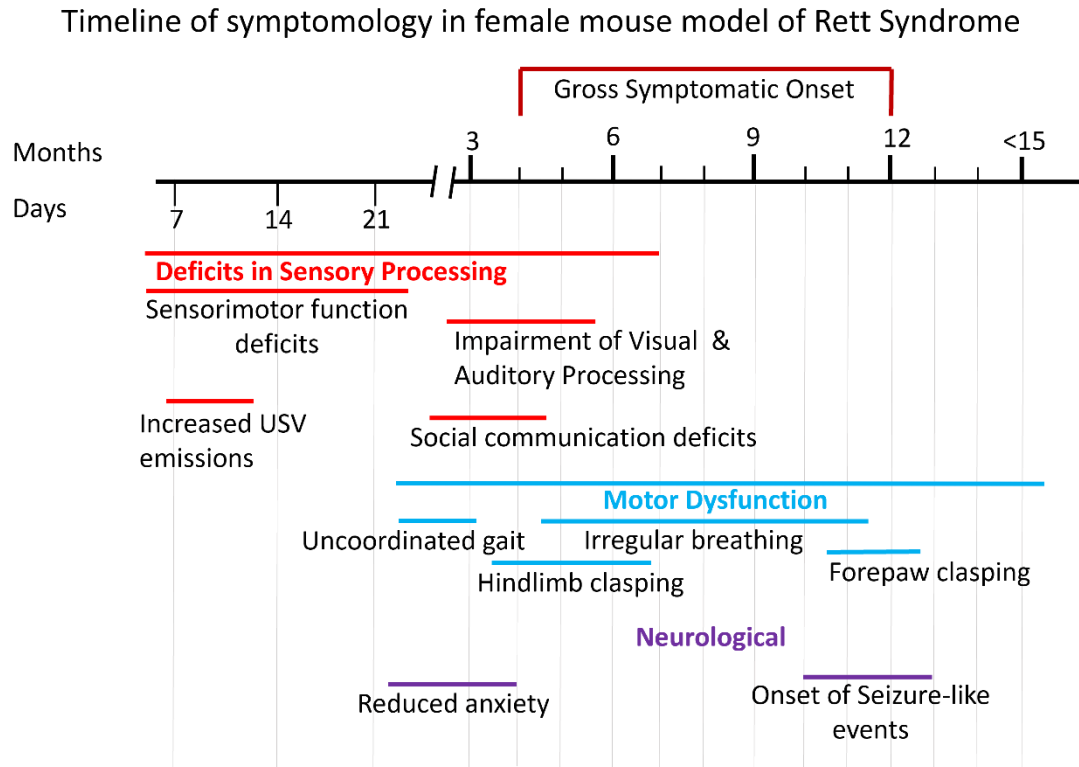


Figure 1.2. Symptomology in female mouse model for Rett Syndrome.

Age is indicated at the top in months and weeks (bottom). Lines indicate the period in which the corresponding stage or symptom appears or has been reported. Red lines indicate the appearance of subtle sensory processing issues early on before the onset of gross motor deficits later in adulthood, shown in blue. Purple lines indicate other neurological symptoms such as reduced anxiety and onset of seizures in late adulthood. Data supported from Guy et al., 2001; Krishnan et al., 2015, 2017; Lau et al., 2020; Picker et al., 2006; Robinson et al., 2013; Samaco et al., 2013; Santos et al., 2007; Stearns et al., 2007).

CHAPTER 2: AGE DEPENDENT SENSORY PHENOTYPES IN A FEMALE MOUSE MODEL FOR RETT SYNDROME

Abstract

Rett syndrome is characterized by an early period of typical development and then, regression of learned motor and speech skills in girls. Loss of MECP2 protein is thought to cause Rett syndrome phenotypes. The specific underlying mechanisms from typical developmental trajectory to regression features throughout life are unclear. Lack of established timelines to study the molecular, cellular, and behavioral features of regression in female mouse models is a major contributing factor. Due to random X-chromosome inactivation, female patients with Rett syndrome and female mouse models for Rett syndrome (Mecp2^{Heterozygous}, Het) express a functional copy of wild-type MECP2 protein in approximately half of all cells. As MECP2 expression is regulated during early postnatal development and experience, we characterized the expression of wild-type MECP2 in the primary somatosensory cortex of female Het mice. Here, we report increased MECP2 levels in non-parvalbumin-positive neurons of 6-week-old adolescent Het relative to age-matched wild-type controls, while also displaying similar levels of perineuronal net expression, mild tactile sensory perception deficits, and efficient pup retrieval behavior. In contrast, 12-week-old adult Het express MECP2 at levels similar to age-matched wild-type mice, show increased perineuronal net expression in the cortex, and display significant tactile sensory perception deficits. Thus, we have identified a set of behavioral metrics and the cellular substrates to study regression during a specific time in the female Het mouse model, which coincide with changes in wild-type MECP2 expression. We speculate that the precocious increase in MECP2 expression within specific cell types of adolescent Het may provide compensatory benefits at the behavioral level, while the inability to further increase MECP2 levels leads to regressive behavioral phenotypes over time.

Introduction

Rett syndrome (RTT) is a neuropsychiatric disorder, predominantly caused by mutations in the X-linked gene Methyl CpG-Binding Protein 2 (MECP2) (Amir et al., 1999a; Hagberg et al., 1983a; Rett, 1966). Most surviving patients with RTT are females who are heterozygous for MECP2 mutations. Diagnostic criteria for RTT include partial or complete loss of acquired purposeful motor skills or spoken language during a regression period in the first few years of life. Additionally, primary sensory processing is disrupted in RTT patients (Buchanan et al., 2019; Djukic et al., 2012; Djukic and Valicenti McDermott, 2012; Key et al., 2019; LeBlanc et al., 2015; Merbler et al., 2020; Nomura, 2005; Peters et al., 2015; Stallworth et al., 2019; Symons et al., 2019). Such disruptions during development and adulthood contribute to motor dysfunction and social communication deficits in girls and women with RTT. Patients typically survive into middle age with periods of plateau and regression throughout life (Bebbington et al., 2008; Buchanan et al., 2019; Charman et al., 2002a; Djukic et al., 2012; Djukic and Valicenti McDermott, 2012; Einspieler and Marschik, 2019; Kerr, 1995; Kirby et al., 2010a; Nomura, 2005). Rodent models recapitulate many of the features of RTT, including sensory processing, social communication deficits, and motor difficulties (Durand et al., 2012; Ito-Ishida et al., 2015; Krishnan et al., 2017a, 2015; Billy Y.B. Lau et al., 2020a; Lee et al., 2017; Lo et al., 2016; Orefice et al., 2016). Though much is known about the characteristics of RTT phenotypes, the underlying cause for regression during various stages of life and the changes in syndromic phenotypes over time is unclear. The prevailing idea is that loss of MECP2 expression and function is the major driver for RTT phenotypes; however, the role of the wild-type MECP2 protein in half of the cells in RTT is unknown.

In RTT, random X-chromosome inactivation leads to mosaic wild-type MECP2 expression in approximately 50% of most major cell types in the body, including neurons (Samaco et al., 2013; Young and Zoghbi, 2004). Due to this inherent mosaicism, individual girls and women with RTT, as well as individual female heterozygous mice (Mecp2 heterozygous, Het), are unique. However, the impact of

mosaic MECP2 expression on cellular function in Het is not fully understood. We hypothesize that the mosaicism in Het contributes to the observed syndromic phenotypes, due to likely compensation and adaptations in individual cells throughout life. In rodents, MECP2 expression increases postnatally, concomitant with the emergence of appropriate critical periods, and is generally thought to be stable in adolescence and adulthood (Braunschweig et al., 2004; Skene et al., 2010; Yagasaki et al., 2018). MECP2 is variably expressed across cell types, with higher expression in GABAergic interneurons in comparison to pyramidal neurons within the same region (Braunschweig et al., 2004; Ito-Ishida et al., 2015; Picard and Fagiolini, 2019; Skene et al., 2010; Takei et al., 2021; Yagasaki et al., 2018). Intriguingly, the status of wild-type MECP2 expression over time, in Het, is unknown. Due to its significant roles in gene regulation (Chahrour et al., 2008; Chen et al., 2015; Fuks et al., 2003; Gabel et al., 2015; Jones et al., 1998; Klose et al., 2005; Lyst and Bird, 2015; Tao et al., 2009; Zhou et al., 2006) and the timing of experience-dependent plasticity (Krishnan et al., 2017a, 2015; Billy Y.B. Lau et al., 2020a; Morello et al., 2018; Noutel et al., 2011; Patrizi et al., 2020; Picard and Fagiolini, 2019), investigation of wild-type MECP2 expression in Het across ages is essential to unravel the mechanisms connecting genotype to phenotype throughout the lifetime of the individual.

Previous work found 6-week-old adolescent Het are thought to not exhibit overt symptoms and 12-week-old adult females are considered early post-symptomatic (Samaco et al., 2013). To specifically address social communication and complex sensorimotor integration, we have used a pup retrieval task, an ethologically-relevant behavior involving primary sensory processing and sensorimotor integration in an untrained task of retrieving scattered pups back to the nest (Beach and Jaynes, 1956; Lonstein et al., 2015; Stern, 1996). Adult Het are inefficient at the pup retrieval compared to age matched wild-type (WT) females (Krishnan et al., 2017a; Stevenson et al., 2021a). Furthermore, adult Het exhibit atypical tactile interactions with pups involving the whiskers and face, with decreased grooming behavior and increased proximal pup approaches (Stevenson et al., 2021a). Together, these results suggest abnormal tactile processing in Het, which could contribute to their inefficient pup

retrieval. Tactile processing is particularly relevant to alloparental care, as sensory cues from offspring are singularly essential for the early retention and maintenance of maternal behavior (Hinde and Davies, 1972; Lonstein and Stern, 1999; Maier, 1963; Morgan et al., 1992; Rosenblatt, 1967; Stern, 1996; Stern and Kolunje, 1989; Stern and Mackinnon, 1978). Thus, we focused on the tactile sensory perception modality in adolescent and adult Het using texture discrimination and object recognition tasks (Antunes and Biala, 2012; Diamond et al., 2008; Orefice et al., 2019, 2016; Wu et al., 2013).

Here, we report the first evidence of a precocious increase of wild-type MECP2 expression within MECP2-expressing cells 6-week-old adolescent Het relative to age-matched WT; these MECP2 expression changes are observed in non-parvalbumin positive (PV+) GABAergic neurons, which exhibit lower levels of MECP2, but not in PV+ GABAergic neurons exhibiting higher MECP2 levels. This cell-type specific expression difference is not maintained in 12-week-old adult Het, suggesting that cells expressing wild-type MECP2 protein in Het are regulated by age-specific mechanisms. This MECP2 expression phenotype in adolescent Het is concomitant with typical structural plasticity, as measured by the expression of specialized extracellular matrix structures ensheathing PV neurons called perineuronal nets (PNNs). Furthermore, systematic, frame-by-frame analyses of texture and object investigation tasks in conjunction with pup retrieval behavior reveal that adolescent Het behave similarly to adolescent WT in both simple tactile and complex social behaviors. Adult Het mice, however, are known to be inefficient at pup retrieval (Krishnan et al., 2017a; Stevenson et al., 2021a) and display overt tactile sensory perception issues, suggesting that observed tactile hyposensitivity in Het is age-related. Based on these findings, we hypothesize that precocious increase of wild-type MECP2 expression in specific cell types of adolescent Het provides compensatory benefits at the behavioral level, while the inability to further increase MECP2 levels with age leads to observed RTT stereotypies over time. Together, these novel results open new directions of exploration on the long-term, cell-type specific roles of MECP2 expression, regulation, and function in Het while emphasizing the need to analyze dynamics of cellular and behavioral phenotypes.

Materials and Methods

Animals

We used the following mouse strains: CBA/CaJ (JAX:000654) and Mecp2heterozygous (Het) (C57BL/6J, B6.129P2(C)-Mecp2tm1.1Bird/J, JAX:003890) (Guy et al., 2001). All animals were group-housed by sex after weaning, raised on a 12/12-hour light/dark cycle (lights on at 7 am) and received food and water ad libitum. Behavioral experiments were performed using Het and female Mecp2WT siblings (WT) at either 6 (adolescent) or 10-to-12 (adult) weeks of age between the hours of 9 am and 6 pm (during the light cycle). All procedures were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Tennessee-Knoxville.

Immunohistochemistry

All animals were perfused with 4% paraformaldehyde (PFA) in 1X PBS immediately after behavior. Extracted brains were stored in 4% PFA overnight at 4°C before storing long-term in PBS. Tissues were processed in cohorts (one WT and one Het) as previously described (Billy Y. B. Lau et al., 2020a). Briefly, coronal brain sections (100 µm thickness) encompassing the entire primary somatosensory cortex (SS1) were collected using a freezing microtome. We denoted the left hemisphere by poking a small hole in the ventral part of the brain. Free-floating sections were blocked with a 10% normal goat serum and 0.5% Triton X-100 in 1X PBS. Blocking solution at half the original concentration was used for antibody incubations. Tissues were incubated with biotin-conjugated WFA lectin (1:500; Sigma-Aldrich), MECP2 primary antibody (1:1,000; Cell Signaling), and PV antibody (1:1,000; Sigma-Aldrich) followed by secondary antibodies conjugated with AlexaFluor-488, Texas-Red, and AlexaFluor-405 (1:1,000; Invitrogen) to label PNNs, MECP2, and PV (if applicable), respectively. Tissues were counterstained with DAPI before mounting section onto slides. All information on antibodies used can be found in **Table 1.1**.

Microscopy and Image Analysis

Image Acquisition

MECP2: Image acquisition of both MECP2 and DAPI signals were performed using a VT-Hawk 2D-array scanning confocal microscope (Visitech intl.) mounted to an IX-83 inverted optical microscope (Olympus). Z-series images with a step interval of 0.5 μm were collected using a 60X objective lens (Nikon) in MetaMorph (Molecular Devices). Settings for each laser channel were determined in a similar manner as previously described for PNNs while utilizing the histogram function in MetaMorph. Acquisition settings were determined using the top 25.0 μm of WT tissues, and these settings were used to image all conditions in the 16-bit range. PV and PNN images (**Figure 2.1**) were acquired in the same manner using a 40X objective lens (Nikon). Images were collected from the Layer IV barrel field subregion (S1BF) in both hemispheres of two sections per brain.

PNN: Single-plane images of coronal tissue sections were collected using a Keyence epifluorescence microscope (Keyence BZ-X710) with a 10X objective and stitched using the BZ-X Analyzer software. We determined exposure settings for each cohort based on Het PNN intensity, as we previously showed atypical increased expression of PNNs in adult Het (Billy Y. B. Lau et al., 2020a). To reduce overexposure, we set the exposure time to the point when the first saturated pixel appeared in the sample, then reduced the exposure time by one unit. This procedure was performed in both hemispheres for all Het sections, and the lowest exposure time occurring most frequently was used to image all sections within that cohort.

Image Processing & Analysis

MECP2: Nuclear expression of MECP2 was quantified using ImageJ (Schneider et al., 2012). For initial background subtraction, a duplicate of the original image was created and processed by applying a median filter with a 32-pixel radius. The filtered image was then subtracted from the original image using the Image Calculator tool to produce 16-bit images for quantification. Mean MECP2 intensity was quantified within individual

nuclei by creating 3.0-5.0 μm maximum intensity projections (16-bit greyscale) within the top 25.0 μm of each tissue. Projections were created using the same image plane for both the DAPI and MECP2 channels and utilized for ROI creation via the Polygon drawing tool and quantification, respectively. DAPI signal, PNN presence or absence, and MECP2-expression status were identified for individual cells using merged tricolor images. Statistical analyses and figures were generated using GraphPad Prism. The percentage of nuclei expressing MECP2 was calculated using the EzColocalization ImageJ plugin (Stauffer et al., 2018). Briefly, the DAPI and MECP2 z-series image stacks were used for reporter channels 1 and 2, respectively, with default threshold algorithms applied. The mean Mander's Colocalization Coefficient (MCC) for MECP2 colocalization with DAPI signal was collected across all imaging planes. This process was repeated for the four images collected for each animal (2 per hemisphere) and averaged to yield an animal specific mean MCC value. To compare MCC values in a standardized manner, the average MCC value for all WT images across both ages was calculated. Then, the mean MCC value for each WT and Het replicate was divided by the WT average MCC and multiplied by 100 to approximate the percentage of nuclei expressing MECP2.

PNN: Images were mapped and regions-of-interests (ROIs) for S1BF were outlined by overlaying the images with coronal maps from Paxinos and Franklin's mouse atlas (4th edition, 2013). The density of high-intensity PNNs were quantified as previously described (Billy Y. B. Lau et al., 2020a) using ImageJ (Schneider et al., 2012). Briefly, high-intensity PNNs were quantified by maximizing the contrast of each image and manually marking the signals that were 80% or more of the original PNN. Density was calculated as the total number of high-intensity PNNs divided by ROI area. We also analyzed the full spectrum of PNN expression in the mapped images. 8-bit histograms specific to the green channel (PNNs) were generated using the Histogram tool in ImageJ. We extracted the distributions of strong PNN expression from all images using two-component Gaussian mixture models (**GMM; see Figure 2.2**) (Bibbings et al., 2019). The means of these distributions (**red traces in Figure 2.2.d**) were used as representative summary statistics in subsequent analyses. We calculated intraclass

correlation coefficients (ICCs) to show that the ratio of inter-individual variance to the total variance was moderate (0.218), such that comparisons on genotypes can be made. We built a linear mixed-effects model to confirm that the inter-individual variability did alter the statistical significance (Yu et al., 2022). The increased variability in Het compared to WT is likely due to natural variation due to random X-chromosome inactivation patterns in Het.

Pup Retrieval Task

Behavioral Paradigm

The pup retrieval task was performed as previously described (Stevenson et al., 2021a). Two 4.5-week-old naïve females (one WT and one Het) with no prior experience of pup retrieval were co-housed with a pregnant CBA/CaJ female (10-12 weeks of age) 3-5 days before birth; naïve females were termed ‘surrogates’ upon cohousing. Once the pups were born (postnatal day 0; P0), we performed the pup retrieval task with the surrogates for six consecutive days. The behavior was performed in the home cage placed inside of a sound- and light-proof box as follows: one surrogate was placed in the home cage with 3-5 pups (habituation; 5 minutes), the pups were removed from the nest (isolation; 2 minutes), and the pups were scattered throughout the home cage by placing them in the corners and center, allowing the surrogate to retrieve pups back to the nest (retrieval; 5 minutes). The nest was left empty if there were fewer than five pups. The assay was performed in the dark and recorded using infrared cameras during all phases of each trial. If all pups were not retrieved to the nest by the 5-minute mark of the retrieval phase, we placed pups back in the nest in preparation for the next surrogate. After the behavior was completed, all mice were returned to the home cage.

Behavior Analysis

All videos were coded so the analyzers were blind to the identity of the mice. Each video was manually scored using a latency index (the amount of time to retrieve all pups back to the nest out of 5 minutes) and the number of errors (surrogate-pup interactions that did not result in successful retrieval). Latency index was calculated as:

$$\text{Latency index} = [(t1 - t0) + (t2 - t0) + \dots + (tn - t0)] / (n \times L)$$

Where n = number of pups outside the nest, t_0 = start time of trial (s), t_n = time of the n th pup's successful retrieval to the nest (s), L = total trial length (300 s). Statistical analyses and figures were generated using GraphPad Prism.

Tactile Perception Tasks

WT and Het underwent a five-day battery of behavioral tests to assay tactile processing through the whiskers (**Figure 2.4**). The behavior chamber used for tactile assays was a 40 cm x 40 cm x 40 cm plexiglass box with two sets of holes in the center of the plexiglass floor for the texture/object inserts. Holes were drilled to be equidistant from the center, with a 4.0 cm spacing between hole sets. Texture discrimination plexiglass inserts were 0.5 cm (thickness) x 4.0 cm (width) x 15.0 cm (height). Rodents use their whiskers to both detect and discriminate between textures and objects (Rodgers et al., 2021). We chose to test the ability of experimental mice to discriminate between textures using both large and small textural differences. Sandpaper textures were designed to slide on and off the inserts to be cleaned between animals. The textures were made of 3M sandpaper of varying grit: a course 60-grit, a medium 120-grit, and a fine 220-grit. Inserts used for novel object recognition were plexiglass spheres (4.0 cm diameter) and plexiglass cubes (64 cm³) (Orefice et al., 2016; Wu et al., 2013). The chamber and inserts were cleaned with MB-10 chlorine dioxide disinfectant and allowed to fully dry before the start of each behavior, and an additional 5-minute period was allotted between tests to eliminate scent biases. All behaviors were performed in the dark and recorded with an infrared camera.

Behavioral Paradigm

Habituation: On days 1 and 2, mice were placed individually into the behavior chamber for 10 minutes to habituate themselves with the new environment.

Texture Discrimination: On days 3 and 4, texture discrimination assays began with a familiarization trial. Experimental mice were individually placed into the chamber with two identical texture inserts and allowed to investigate for 10 minutes. Afterwards, the mouse was returned to the home cage for 5 minutes. During this time, textures and

inserts for familiarization were replaced with new sets of inserts and textures for use in the testing trial. After 5 minutes, the mouse was returned to the behavior chamber for a second 10-minute trial (testing phase). Familiarization phases of each behavior were performed using two coarse texture inserts. During testing phase on days 3 and 4, we replaced one insert with a medium and fine texture insert, respectively.

Novel Object Recognition: On day 5, a novel object recognition assay was performed similar to the texture discrimination assay with minor changes. The familiarization trial was performed using two plexiglass cubes. For the testing trial, one plexiglass cube was replaced by a plexiglass sphere.

Behavior Analysis

Behaviors for both texture and object investigation were manually scored using Datavyu software (Datavyu Team, version 1.3.7, 2014). All analyzers were blind to the age and genotype of the mice. The videos were scored for the time intervals in which the mouse interacted with the novel and familiar textures/objects throughout the 10-minute testing trial. We classified interactions into three categories, as shown in Table 1.1, based on pilot observations and previous studies (Orefice et al., 2016; Pacchiarini et al., 2017; Wu et al., 2013). We excluded mice from analysis if the investigation time of textures/objects during a given trial did not exceed 2 seconds (Orefice et al., 2016; Patrick Wu and Dyck, 2018; Wu et al., 2013) (Table 1.2). After manual scoring, interaction sequences for each animal were extracted from Datavyu for quantitative analyses. We classified number of contacts, total investigation time, and duration per interactions with textures/objects into whisker, forepaw, and whole-body climbing. Our results are reported as raw number of contacts, raw total investigation times, and raw duration per interaction split by interaction type, age, and genotype. We conducted unpaired Mann Whitney-U tests to determine statistical significance between genotypes and across ages within the same genotype. Statistical analysis and data visualization were performed in R studio.

Results

Wild-type MECP2 expression is dynamically regulated in Het over age and cell types

MECP2 protein expression varies in different cortical cell types (Ito-Ishida et al., 2015; Takei et al., 2021). However, it is currently unknown if cell-type specific MECP2 expression is stable over age or if wild-type MECP2 expression in Het is similar to age-matched WT controls. Het expression of wild-type MECP2 was reported to be decreased relative to age-matched WT females; however, the brain region(s) and the specific ages of mice used in this investigation were not reported (Braunschweig et al., 2004). Thus, we performed immunofluorescent staining and confocal microscopy to first characterize MECP2 expression within individual nuclei of naïve WT and Het female mice during adolescence and adulthood. We chose to investigate expression within Layer IV of the barrel field subregion of the primary somatosensory cortex (S1BF), the cortical input layer from the whiskers, due to its importance in tactile sensory perception. Previously, we reported that adult Het exhibit dysregulation of PV+ GABAergic neurons in the auditory and primary somatosensory cortices (Krishnan et al., 2017a; Billy Y.B. Lau et al., 2020a; Billy Y. B. Lau et al., 2020a). Particularly, adult Het mice exhibit increased and atypical expression of perineuronal nets (PNNs) in the S1BF (Billy Y. B. Lau et al., 2020a), extracellular matrix structures mainly surrounding cortical PV+ GABAergic interneurons to structurally restrict plasticity (de Vivo et al., 2013; Hartig et al., 1992; Krishnan et al., 2017a; Pizzorusso et al., 2002; Ueno et al., 2018). Thus, we focused MECP2 expression analysis in PNN+ PV nuclei and neighboring PNN-negative nuclei (termed non-PV) within S1BF (**Figure 2.1.a**).

PNN+ PV nuclei of WT exhibited a roughly 3-fold higher level of MECP2 than in non-PV nuclei in Layer IV S1BF across adolescence and adulthood (**Figure 2.1.a, b**). This fold increase is higher than the 1.25-fold increase reported in Layers II/III (Ito-Ishida et al., 2015). We observed significant increases in MECP2 expression between adolescence and adulthood in WT females across cell types (**Figure 2.1.c, d, f, g**). In Het, approximately 50% of the nuclei (identified by DAPI staining) expressed wild-type

MECP2 protein at both ages (**Figure 2.1.e**), in agreement with previous reports (Samaco et al., 2013; Young and Zoghbi, 2004). Strikingly, adolescent Het non-PV nuclei expressed significantly higher levels of MECP2 than age-matched WT, while adult Het non-PV nuclei did not exhibit such a difference (**Figure 2.1.c, f**). In PV nuclei, MECP2 expression was significantly increased between adolescence and adulthood in both WT and Het (**Figure 2.1.d, g**), without significant differences between genotypes within ages. Together, these results show that Het displays a precocious increase of wild-type MECP2 levels, as well as an age-dependent failure to further increase wild-type MECP2 expression levels on par with WT females, within specific cell types.

No changes in perineuronal net expression in S1BF of adolescent Het

MECP2 regulates the expression of perineuronal nets (PNNs), specialized extracellular matrix structures that structurally restrict changes in synaptic plasticity (Carstens et al., 2021, 2016; de Vivo et al., 2013; de Winter et al., 2016; Deepa et al., 2002; Dityatev et al., 2007; Donato et al., 2013; Durand et al., 2012; Gogolla et al., 2009, 2006; Hou et al., 2017; Kosaka and Heizmann, 1989; Krishnan et al., 2017a, 2015; Morello et al., 2018; Nakagawa et al., 1986; Patrizi et al., 2020; Pizzorusso et al., 2002; Sigal et al., 2019; Thompson et al., 2018; Ueno et al., 2017). Adult Het show atypical increases in the expression of mature, high intensity PNNs in S1BF and the auditory cortex (Krishnan et al., 2017a; Billy Y. B. Lau et al., 2020a). PNNs are formed on PV neurons from an array of secreted proteins sourced from multiple cell types (Bignami et al., 1992; Carulli et al., 2007, 2006; Kwok et al., 2010; Miyata and Kitagawa, 2017). Thus, we hypothesized that the increased MECP2 levels within non-PV cells would regulate adolescent Het PNN expression to typical WT levels in a non-cell autonomous manner.

As we found nuanced changes in latero-medial axis and a hemisphere-specific difference in PNN expression in adult females (Billy Y. B. Lau et al., 2020a), we used serial sectioning, immunofluorescent staining, and epifluorescence microscopy to manually quantify high-intensity PNN expression across the entire S1BF (**Figure 2.2.a, b**). We found no significant difference in high-intensity PNN density between adolescent WT and Het across the entirety of the S1BF (**Figure 2.2.c**). Additionally, we did not find

differences in PNN densities between hemispheres of either adolescent genotype. (WT-L = 12.845 ± 1.607 # PNNs/mm², WT-R = 18.315 ± 1.016 , n = 307 images, 6 animals; Het-L = 13.241 ± 0.380 , Het-R = $14.932 \pm .347$, n = 313 images, 6 animals).

Differing intensities of PNNs are thought to represent levels of maturity of that PV network in early developing postnatal cortex. However, it is unclear if the same standards hold true for adolescent cortices. Thus, we performed additional analyses using an automated, unbiased approach to select all pixels that represent PNN expression (**Figure 2.2.d, red distributions in top two panels; see Methods**) over background (**Figure 2.2.d, blue distributions in top two panels**). We observed that PNN expression in all tissues had multimodal pixel intensity distributions, which consistently included a bell-shaped distribution at higher intensities (**Figure 2.2.d insets**). This distribution represents specific cortical layers with strong PNN expressions (Layers II/III, IV, and V) of interest for quantification. Models consistently captured the distributions of PNN expression in the expected intensity region (**Figure 2.2.d, red**). By analyzing the mean distribution of these red traces, we observed no significant differences in PNN signal between adolescent WT and Het using a linear mixed-effects model with cohort as a variable (**Figure 2.2.d, bottom panel**). The intraclass correlation coefficient across all cohorts was moderate (0.218), demonstrating that variation within the dataset was significantly driven by natural variation due to random X-chromosome inactivation, over animal-to-animal variability (See Materials and Methods). Together, these results suggest that MECP2 expression in non-PV cells affects PNN expression in PV cells in an age-dependent manner.

Adolescent Het perform a pup retrieval task with comparable efficiency to age-matched WT

Adult Het display sensory deficits while performing the complex social behavior of alloparental care, as measured by the pup retrieval task (Krishnan et al., 2017a; Stevenson et al., 2021a). Since adolescent Het expressed more MECP2 in non-PV nuclei and expressed PNNs at WT levels, we predicted they would perform efficiently in the pup retrieval task (**Figure 2.3**). Adolescent Het and WT improved at pup retrieval

across six consecutive days of testing as measured by time to retrieve scattered pups to the nest (**latency index; Figure 2.3.a, a'**) and number of errors (**Figure 2.3.b, b'**). Additionally, adolescent Het retrieved pups with a similar efficiency as adolescent WT females. Adult Het are comparably inefficient at this same task (Stevenson et al., 2021a). Cumulatively, these results suggest that the increased MECP2 levels within non-PV nuclei, as well as typical PNN expression associated with PV+ interneurons, provide a molecular mechanism for compensatory behavioral adaptations during adolescence which are lost in older females, resulting in a regressive behavioral phenotype.

Het display regression in whisker tactile sensory behavior between adolescence and adulthood

Pup retrieval involves initial perception of sensory cues, multisensory integration, and motor coordination involving multiple neural circuits. Here, we chose to focus specifically on the single sensory perception modality of whisker tactile perception due to its connection to S1BF. We performed standard texture discrimination and object recognition tasks on adolescent and adult WT and Het and analyzed their behavior frame-by-frame using DataVyu software (**Figure 2.4**).

Standard representation of percentage of time spent with different textures did not change in adolescent females during texture investigation (WT: 50.14% $\pm$ 4.344, n=9; Het: 55.60% $\pm$ 4.116, n=10; one-sample t-test with 50% chance) or during object investigation (WT: 47.74% $\pm$ 4.263, n=9; Het: 50.33% $\pm$ 3.572, n=10; one-sample t-test with 50% chance). Percentage of time spent with textures did not change in adult WT or Het female mice (WT: 57.08% $\pm$ 3.356, n=9; Het: 51.59% $\pm$ 3.531, n=8; one-sample t-test with 50% chance) and during object investigation (WT: 51.67% $\pm$ 4.477, n=9; Het: 57.26% $\pm$ 4.511, n=8; one-sample t-test with 50% chance). Thus, we refrain from drawing conclusions about preferences of the female WT and Het mice or their memory capabilities here.

We chose to perform a systematic analysis of behavioral videos, as mice interact with textures and objects with high speed and rarely linger during investigations. We found that both adolescent WT and Het females predominantly investigated textures with

whiskers and interacted with similar number of contacts (**Figure 2.5.a, b**). However, though the total interaction time with textures did not differ significantly between adolescent WT and Het (**Figure 2.5.c**), the duration per whisker interaction was significantly higher in Het (**Figure 2.5.d**).

We performed similar analyses with novel object recognition to determine if adolescent Het are deficient in other forms of tactile sensory-dependent tasks. Adolescent WT and Het used their whiskers, forepaws, and whole-body climbing to investigate objects (**Figure 2.6.a**). Interestingly, adolescent Het made significantly fewer whisker contacts (**Figure 2.6.b**) and spent similar total time investigating objects (**Figure 2.6.e**) compared to age-matched WT. There was no significant difference in the number of contacts or time spent investigating objects using forepaws (**Figure 2.6.c, f**) and whole-body climbing (**Figure 2.6.d, g**). Similar to texture investigation, adolescent Het interacted significantly longer per interaction using whiskers (**Figure 2.6.h**), but not forepaws or whole-body climbing (**Figure 2.6.i, j**), compared to WT. Together, the increased duration of interactions when interacting with textures and objects suggests the beginnings of an atypical whisker perception in adolescent Het.

On the contrary, adult Het displayed significant whisker hyposensitivity in texture discrimination compared to adult WT (**Figure 2.7**). Examples of frame-by-frame sequence analysis showed that non-whisker interactions (forepaw and whole-body climbing) were interspersed with whisker interactions in adult Het (**Figure 2.7.a**). Compared to WT, adult Het used whisker to make significantly more contacts, spent more time investigating textures, and had longer durations per whisker interaction with textures (**Figure 2.7.b, e, h**). Adult Het also used forepaws and whole-body climbing to make significantly more contacts, spent more time investigating, and had longer time per interaction with textures than adult WT (**Figure 2.7.c-d, f-g, i-j, respectively**). These findings are similar to previous studies in whiskerless and barrel-less mice (Guic-Robles et al., 1992; Guic-Robles et al., 1989) and suggest adult Het are seeking compensatory tactile sensation due to deficits in whisker sensation (hyposensitivity). Furthermore, this systematic analysis captured the individual variation expected in adult

Het phenotypes. Adult Het 3 and 5 (**Figure 2.7.b-d, h-j, light blue and yellow colors respectively**) were similar to adult WT in number and duration of contacts with whiskers, and no interactions with forepaw or whole-body, suggesting a likely typical functionality within S1BF of the respective animals. Adult Het 6 and 7 (dark blue and deep orange respectively) performed forepaw interactions, but not whole-body climbing (**Figure 2.7.c, d, f, g, i, j**), suggesting minor sensory processing deficits in S1BF which might be compensated for by appropriate tactile perception within the forelimb region of the primary somatosensory cortex. The other five adult Het were most different from adult WT in their texture interactions, likely to have profound deficits in neural processing in S1BF and neighboring SS1 subregions.

Surprisingly, adult Het had milder phenotypes while interacting with objects (**Figure 2.8**). Unlike texture investigations in adults, both adult WT and Het also used forepaws and whole-body climbing to interact with objects (**Figure 2.8.a**). We did not observe significant differences in both the total number of contacts with objects and the total time spent investigating objects between adult WT and Het during whisker, forepaw, or whole-body climbing interactions (**Figure 2.8.b-g**). Adult Het spent significantly longer time per interaction with objects using whiskers (**Figure 2.8.h**), but not forepaws or whole-body climbing (**Figure 2.8.i, j**). Taken together, these results point to the beginnings of a regressive phenotype associated with specific tactile sensory perception in 6-week-old female Het that progresses to a severe, context-dependent tactile hyposensitivity by 12 weeks of age.

Context-specific developmental progression in WT and regression in Het

To develop a baseline in tactile performance of females in both adolescence and adulthood, we compared texture- and object-associated phenotypes within genotype and across age. Compared to adolescent WT, we found that adult WT spent significantly less time with textures (**Figure 2.9.a, b**), while the number of contacts remained similar across ages (**Figure 2.9.c**). On the other hand, adult Het spent significantly more time with textures and more numbers of contacts (**Figure 2.9.d, f**) compared to adolescent Het, while the duration per contact remained similar (**Figure 2.9.e**). Together, these results suggest a progressive perception via whisker tactile

interactions over age in WT, while Het display regressive inefficiencies by these same parameters. Interestingly, neither WT nor Het showed any significant age-specific changes in interactions with objects (**Figure 2.10.a-f**), suggesting that female mice may use different strategies while interacting with finer textures and objects.

Discussion

Disrupted sensory processing is a contributing factor to many of the behavioral phenotypes in neurodevelopmental disorders (Rais et al., 2018; Schaffler et al., 2022). Here, we use established models of whisker tactile sensory perception to discover the impact of mosaic MECP2 expression in identified cell types across developmental stages in a female mouse model for Rett syndrome (**Figure 2.11**). These results set the stage to explore the mechanistic links between age-dependent MECP2 expression levels to neural circuits involved in sensory perception during simple and complex behavioral tasks within Het. Two major observations are of importance to the RTT field: (1) wild-type MECP2 protein expression is regulated in an age- and cell-type specific manner in Het, providing an unexplored angle for therapeutic intervention in the Het brain via dynamic protein regulation, and (2) female Het mice have typical functioning capacity which is lost with age, suggesting that the Het brain is capable of cellular and behavioral plasticity, even past early infancy. Deep phenotyping at cellular and behavioral levels is necessary in this important preclinical model of Rett syndrome to aid in better therapeutic treatments.

Aberrant homeostasis of MECP2 in late adolescence as a catalyst for the transition from pre-symptomatic to symptomatic RTT female mice

One of the most intriguing results here is the observed differences in MECP2 expression within nuclei of Layer IV S1BF non-PV cells in adolescent Het compared to age-matched WT. The observation of cell-type specific and age-dependent MECP2 expression dynamics within Het provides new avenues for consideration in RTT pathogenesis. To date, Rett syndrome is thought to be caused by the lack of functional MECP2 in roughly half of all cells. We speculate that the transient fluctuation in MECP2 levels within the ~50% of the cells expressing the wild-type protein is an additional

direction to consider. The precocious increase in MECP2 within non-PV nuclei of Het may be a cell-autonomous mode of compensation for the lack of MECP2 protein in surrounding cells. Over time, however, the ability to further increase MECP2 expression levels in non-PV nuclei, as observed in all WT cells as well as Het PV cells, is lost.

Thus, although the genotype and the cellular status of the Het remain the same throughout life, their MECP2 expression levels are changing in a cell-type specific manner, suggesting built-in homeostatic mechanisms to regulate MECP2 expression. This could be a basis for normal development in infancy for girls diagnosed with RTT, and then regression at different times throughout life (Neul, 2019). It is equally intriguing that MECP2 expression in PV neurons, with higher basal levels than in non-PV cells, exhibit typical increases comparable to WT females across age. Yet, the underlying mechanisms regulating MECP2 expression levels within specific cell types across age remain unclear. Sorting specific cell types and measuring MECP2 protein content, with regional and age specificities, are necessary to establish an extensive expression baseline in Het, as well as to determine how MECP2 expression changes with experience within context-specific circuitry.

In female Het mice, the precocious, cell-type specific increases in MECP2 expression coincides with pubertal changes. In female WT mice, onset of puberty is accelerated by leptin (Ahima et al., 1997; Chehab et al., 1997) and ovarian hormones that regulate the organization and maturation of cortices by regulating inhibition (Clemens et al., 2019; Piekarski et al., 2017). This line of research is important as girls with RTT enter puberty early and reach menarche later, and body-mass index affects pubertal timing (Killian et al., 2014). If Het mice also undergo pubertal deviations from their WT littermates, atypical neuroendocrine signaling would affect Het females as they transition into adulthood, in terms of both steroid-dependent organization of circuitry and MECP2 expression.

MECP2 expression changes in appropriate primary sensory cortices in early developmental critical periods (He et al., 2014; Krishnan et al., 2015; Skene et al., 2010; Yagasaki et al., 2018) and in response to different stimuli (Martínez de Paz et al., 2019;

Yagasaki et al., 2018). MECP2 expression in adolescence and adulthood is thought to be stable. However, systematic studies with cell-type specific fluorescent-assisted cell sorting over brain regions, ages, and experimental conditions are required to determine the extent of the dynamic range of MECP2 expression. Does the expression level of MECP2 matter for its nuclear functions of binding methylated DNA and regulating gene expression? Though not thoroughly investigated at the mechanistic level, increasing MECP2 expression in transgenic over-expression rodent lines (Collins et al., 2004) or deleting MECP2 in specific cell types also largely results in Rett syndrome-like phenotypes at behavioral and cellular levels (Bhattacharjee et al., 2017; Chapleau et al., 2009; Goffin et al., 2014; He et al., 2014; Ito-Ishida et al., 2015; Krishnan et al., 2017a; Meng et al., 2016; Mossner et al., 2020). However, how MECP2 expression in neighboring cells are affected is unknown in these manipulations. Moreover, considering the possibility of compensatory changes in MECP2 expression, it is critical to determine the impact of these manipulations on MECP2 expression in unmanipulated, non-target cells in an age-specific manner. Furthermore, we speculate that such compensatory mechanisms at the protein level plays crucial roles in other X-linked neurodevelopmental disorders (Brand et al., 2021; Santos-Rebouças et al., 2020) metabolic disorders (Juchniewicz et al., 2021).

Perineuronal nets and PV-pyramidal networks in Mecp2-deficient mouse models

Germline deletion of Mecp2 from all cell types, as well as conditional deletion of Mecp2 from both PV neurons and forebrain pyramidal neurons, result in myriad of cellular and behavioral phenotypes, particularly in male mice (Chao et al., 2010; Gemelli et al., 2006; Ito-Ishida et al., 2015; Krishnan et al., 2015; Morello et al., 2018; Mossner et al., 2020; Patrizi et al., 2020). An altered PV-network configuration interferes with experience-dependent plasticity mechanism in the brain and is associated with defective learning and plasticity (Donato et al., 2013; Krishnan et al., 2015; Patrizi et al., 2020). Adult Het mice have atypical increased expression of PNNs in the auditory cortex (Krishnan et al., 2017a) and specific subregions of the primary somatosensory cortex (Billy Y. B. Lau et al., 2020a). Typically, pup vocalizations trigger suppression of PV+ GABAergic neuronal responses and a concomitant disinhibition in deep-layer pyramidal neurons in the auditory cortex of adult WT surrogates (Lau et al., 2020a). In

adult Het surrogates, the increased perineuronal net expression interferes with the synaptic plasticity of PV+ GABAergic neurons, resulting in a lack of disinhibition of pyramidal neurons (Lau et al., 2020a) and coinciding with poor pup retrieval performance (Krishnan et al., 2017a). It is currently unknown if similar mechanisms also occur in the primary somatosensory cortex, and if so, whether they occur in an age-dependent manner, which would ultimately lead to atypical tactile processing in Het. Studies are underway to determine how PNN expression in S1BF contributes to tactile sensory perception.

Systematic analysis of behavioral assays to study tactile sensory perception in female mice

Traditionally, texture discrimination and object recognition assays are reported as “percent novelty recognition” or a “discrimination index” calculated as total time spent investigating the novel texture/object divided by the total time spent investigating both textures/objects (Antunes and Biala, 2012; Ennaceur et al., 2005; Ennaceur and Delacour, 1988). This analysis normalizes the time spent investigating across individual animals to control variation in exploration time. Based on these metrics, a mixed result of tactile hyper- and hyposensitivity has been previously reported in models of MECP2 deficiency (Chao et al., 2010; Gemelli et al., 2006; Ito-Ishida et al., 2015; Orefice et al., 2016). It is hypothesized that mice will spend more time with novel texture/object due to their innate nature of curiosity and their ability to remember old or novel objects/textures from such interactions. However, a growing body of literature shows that free-moving rodents do not have a preference for novelty (Antunes and Biala, 2012; Cohen and Stackman Jr., 2015; Ennaceur, 2010). Mice and rats show preference for familiar objects rather than novel objects (Ennaceur, 2010). Adult C57BL/6 male mice show novelty object preference, whereas C57BL/6 adult females show no preference between a familiar and novel object (Frick and Gresack, 2003), consistent with our findings. Thus, to reconcile these differences in the literature and establish first order metrics for analysis, we performed a systematic frame-by-frame analysis of interactions with textures and objects for adolescent and adult female C57BL/6 mice. By analyzing the number and time of interactions, we found that adult WT females spent less time interacting with textures compared to adolescent WT females. On the other hand, adult

Het females interacted more overall and longer than adolescent Het females, suggesting a regressive tactile hyposensitivity phenotype in adult Het.

Regression in RTT animal models

Previous work using a female rat RTT model has reported a regression in a learned forepaw skill over the course of weeks (Veeraragavan et al., 2016). Regressive breathing abnormalities have also been shown using a conditional MECP2 knockout male mouse model (Huang et al., 2016). Previously, we have proposed that adult female Het show regression in maintaining pup retrieval efficiency over few days (Stevenson et al., 2021a). While these studies report regression in parasympathetic and motor function, our work here is the first to report a regressive hyposensitivity phenotype in tactile perception between adolescence and adulthood in a female mouse model for RTT. An alternative explanation for increases in tactile interactions from adolescence to adulthood in Het is the emergence of repetitive obsessive-compulsive disorder-like phenotypes over time, as observed in mouse models of this disorder (Ahmari, 2016; Alonso et al., 2015; Mitra and Bult-Ito, 2021). Adult Het perform repetitive jumping during early days of pup retrieval which could be indicative of novelty- or stress-induced stereotypies in a subpopulation of adult Het (Stevenson et al., 2021a). It is currently unclear if the increased frequency and longer durations of interactions in adult Het are a form of repetitive obsessive-compulsive behavior. Clozapine injection has been shown to exacerbate such repetitive phenotypes (Kang et al., 2020) and can be utilized to test this hypothesis in the future.

Rehabilitation strategies

Early interventions aimed at increasing neuronal activity mitigate disease onset in both male and female mouse models for Rett syndrome. Early administration of Ampakine CX546, a positive modulator of AMPA receptors, from postnatal days 3-9 in male *Mecp2*-null mice enhances neuronal activity, prolongs lifespan, delays disease progression, and rescues both motor abilities and spatial memory (Scaramuzza et al., 2021). In Het females, repetitive motor training enhances neuronal activity of task-specific neurons and improves both spatial memory and motor function well into adulthood (Achilly et al., 2021). Adolescent and adult Het improve in the pup retrieval

task with repeated trials over days, in contrast to non-consecutive days of training (Krishnan et al., 2017a; Stevenson et al., 2021a). Early and repetitive trainings delay the onset of disease phenotypes and improve the latency to survival in Het, supporting the plastic capacity of the adolescent Het brain (Achilly et al., 2021). We speculate that wild-type MECP2 expression in Het is affected by these trainings, especially at earlier ages, resulting in activation of compensatory pathways which lead to appropriate behavioral adaptations. Further work is needed to identify therapeutic and rehabilitative strategies that target MECP2 maintenance within the female Het brain for mitigating disease progression throughout life.

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Appendix

Table 2.1. Antibodies used in Chapter 1

Antibody Type	Antibody Name	Immunogen	Codes	Host / Clonality	Source	Dilution	Link for References
Primary	Lectin from <i>Wisteria floribunda</i> – Biotin Conjugate	N/A	RRID:AB_2620171 Cat #: L1516	N/A	Sigma–Aldrich, St. Louis, MO, USA	1:500	link
	MeCP2 (D4F3) XP®	Human MECP2 C-terminus	RRID:AB_2143849 Cat #: 3456	Rabbit Monoclonal	Cell Signaling Technology, Danvers, MA, USA	1:1000	link
	PV	Frog Muscle Parvalbumin	RRID:AB_477329 Cat #: P3088	Mouse Monoclonal	Sigma–Aldrich, St. Louis, MO, USA	1:1000	link
Secondary	Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 405	Gamma Immunoglobins Heavy and Light chains	RRID:AB_2890536 Cat #: A48255	Goat Polyclonal	Invitrogen, Waltham, MA, USA	1:1000	link
	Streptavidin, Alexa Fluor™ 488 Conjugate	N/A	RRID:AB_2315383 Cat #: S32354	N/A	Invitrogen, Waltham, MA, USA	1:1000	link
	Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Texas Red-X	Gamma Immunoglobins Heavy and Light chains	RRID:AB_2556779 Cat #: T-6391	Goat Polyclonal	Invitrogen, Waltham, MA, USA	1:1000	link
Other	DAPI	N/A	Cat #: D9542	N/A	Sigma–Aldrich, St. Louis, MO, USA	1:1000	link

Table 2.2 Definitions of parameters used to score behaviors.

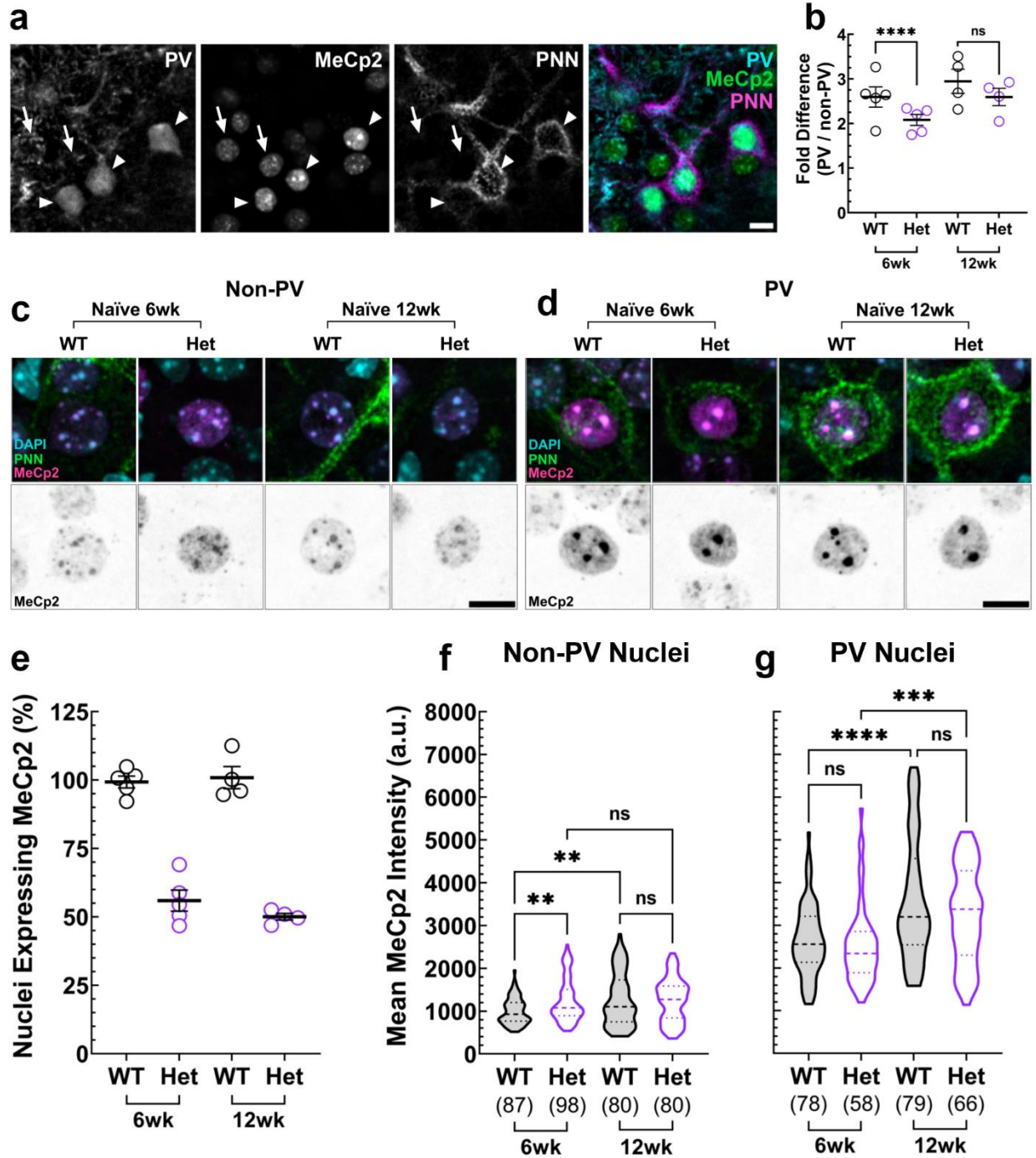
Whisker	- Mouse within 3 cm of the object (due to typical whisker length) and actively exploring around texture/object
Whole-body climbing	- All four paws are off the ground and animal is climbing the texture/object
Forepaw	- Touching the object/texture with forepaws while the hind limbs are contacting the ground

Table 2.3 Number of animals included and excluded from analysis. Animals were excluded from analysis due to technical difficulties with video lagging/skipping or no interactions with textures.

		Included in analysis			Excluded from analysis	
		WT	Het		WT	Het
12 WO	Texture Discrimination Day 1 (Trial 1 & 2)	8	7		1	1
	Texture Discrimination Day 2 (Trial 3 & 4)	9	8		1	0
	Novel Object Recognition	9	8		0	0
6 WO	Texture Discrimination Day 1 (Trial 1 & 2)	8	8		1	2
	Texture Discrimination Day 2 (Trial 3 & 4)	9	10		1	0
	Novel Object Recognition (Trial 1 & 2)	9	10		0	0

Figure 2.1. MECP2 is expressed differentially between cell types in S1BF of adolescent and adult WT and Het.

(a) Representative 40X confocal images from Layer IV S1BF showing PNN colocalization with PV in MECP2+ nuclei. Arrows (non-PV) and arrowheads (PV) point to representative nuclei for quantification. Scale bar = 10 μ m. **(b)** Mean intensity of MECP2 protein from PV and non-PV nuclei is represented as a fold difference (PV:non-PV ratio; number of replicates same as reported for Figure 2.1.f). **(c, d)** Representative nuclei stained with DAPI and MECP2 from non-PV (PNN-negative cells) **(c)** and PNN-positive parvalbumin (PV) interneurons **(d)** in adolescent and adult naïve WT and Het show that MECP2 intensity is higher within PV nuclei across ages, and non-PV nuclei of adolescent (6wk) Het appear more intense compared to age-matched WT or adult (12wk) Het. Scale bars = 10 μ m. **(e)** Het express MECP2 in approximately 50% of nuclei, compared to WT across ages, as measured by percentage of MECP2-positive nuclei in adolescent and adult WT and Het (n = 2 images per hemisphere per brain). **(f-g)** Violin plots of the mean intensity of nuclear MECP2 within **(f)** non-PV and **(g)** PV nuclei. MECP2 expression increased significantly with age in non-PV nuclei of WT, while MECP2 expression in non-PV cells remained consistent across ages. Compared to age-matched WT control, 6-week-old Het showed a significant increase in MECP2 expression in non-PV cells, while 12-week-old Het did not. In PV nuclei, MECP2 expression increased significantly with age within both genotypes; there were no significant differences between WT and Het MECP2 expression at either age. Respective ages are indicated on the x-axes, and the number of nuclei from which data was collected is shown in parentheses for each condition (n = 5 animals per genotype at adolescent age; n = 4 animals per genotype at adulthood). Kruskal-Wallis followed by Uncorrected Dunn's test: ns = not significant; **p < 0.01, ***p < 0.001. **(b, e)** Mean $\pm$ SEM are shown.



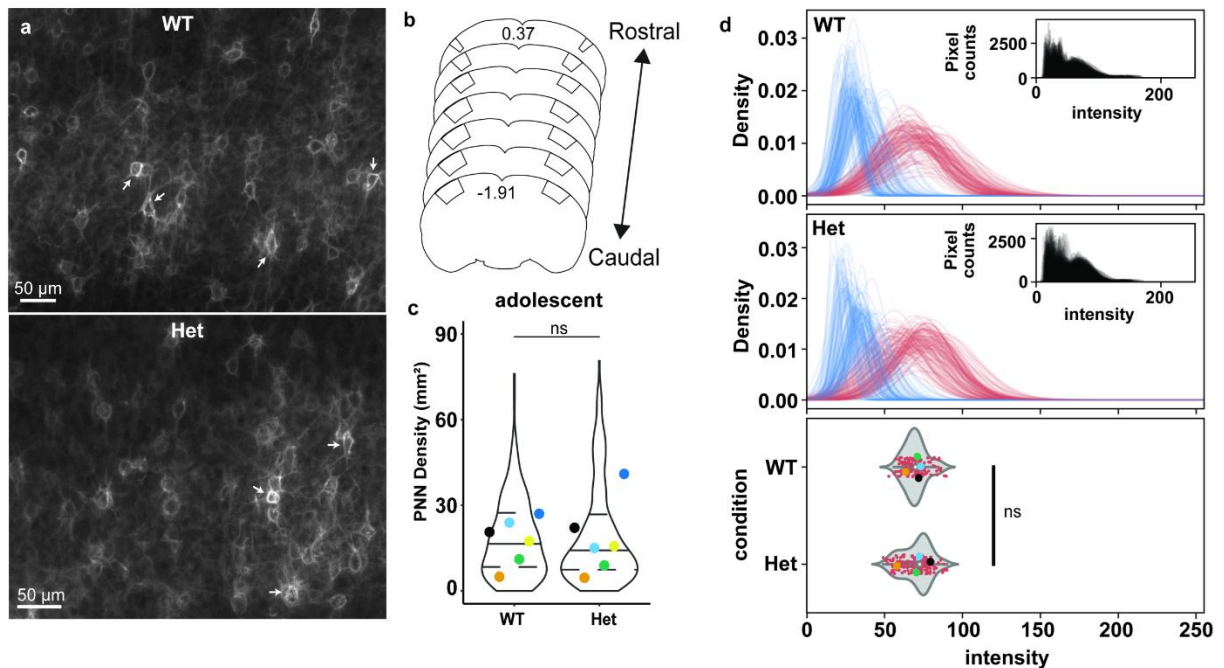


Figure 2.2. Adolescent Het show similar PNN density as WT within S1BF.

(a) Representative epifluorescent 10x micrographs of PNNs in S1BF of adolescent WT and Het. Arrows indicate high-intensity PNNs quantified for analysis. **(b)** Schema of coronal mouse brain sections, depicting rostral (Bregma: 0.37 mm) to caudal (Bregma: -1.91 mm) regions of S1BF (boxes) analyzed for this study. Coordinate maps are based on Paxinos and Franklin's mouse brain atlas, 4th edition. **(c)** Statistical analysis of manually counted high-intensity PNNs revealed that the density is similar between adolescent WT and Het. Each circle represents the average high-intensity PNN density of each brain, superimposed on violin plots showing median with distribution of all sections. Individual colors represent cohorts processed and analyzed together. WT: $n=307$ images, 6 animals. Het: $n=313$ images, 6 animals. Mann-Whitney test: ns = not significant. **(d)** All intensity analysis of PNN expression. The top two panels show two distributions of PNN intensity obtained with a two-component Gaussian mixture model for each image (see Methods). The distributions with higher means (red traces) were used for subsequent analyses, while background signal (blue traces) was excluded. Each trace represents PNN intensity distribution of an image. Insets show histograms of raw intensities for pixels of all images (135 WT and 140 Het). Bottom panel: statistical analysis revealed no significant difference in all PNN intensity between adolescent WT and Het. The graph shows the distribution of the means (small red dots and violin plots) of the red traces in top panels. Large dots show aggregated mean of the red distributions for each color-coded cohort. Box plots within violin plots show 25th, 50th and 75th quartiles. Linear mixed effect model and t-test: $p > 0.05$.

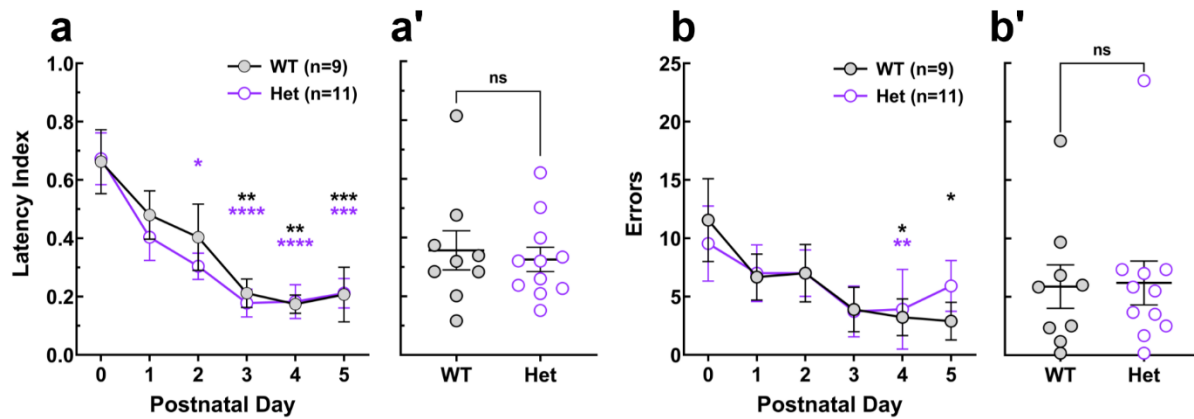


Figure 2.3. Adolescent Het perform pup retrieval comparable to WT.

(a-a') Mean latency index of pup retrieval behavior for each testing day **(a)** and averaged per animal across all testing days **(a')** showed significant improved retrieval across testing days for both WT and Het, and no significant difference in average latency between genotypes. **(b-b')** Mean number of errors during pup retrieval for each testing day **(b)** and averaged per animal across all testing days **(b')** showed same result as latency index. (all panels) Mean $\pm$ SEM are shown. (a, b) Kruskal-Wallis followed by Uncorrected Dunn's test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Color * = within genotype, significance between P0 and P-day. (a', b') Mann-Whitney test: ns = not significant.

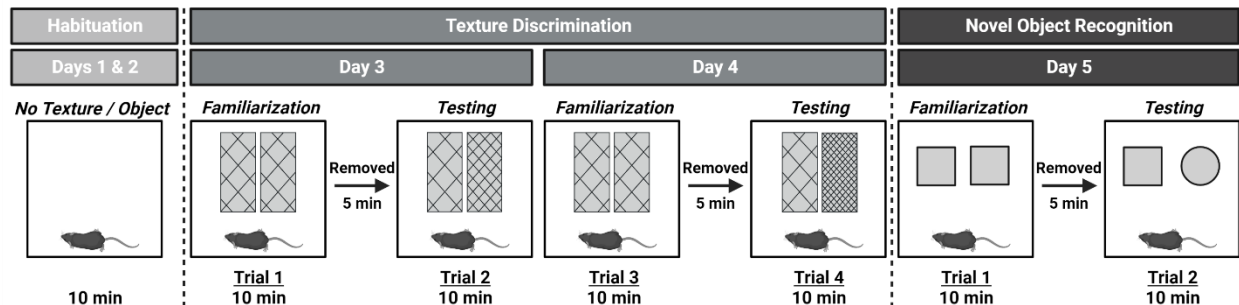


Figure 2.4. Tactile behavior schema.

Behavior tests begin with two days of habituation to introduce the mice to the behavioral box set up. On each days of texture discrimination (days 3 and 4), individual mouse undergoes a 10-minute familiarization trial followed by a 10-minute testing trial, with varied sandpaper grits. On day 5, objects are used instead of textures for novel object recognition task. See Methods for detail.

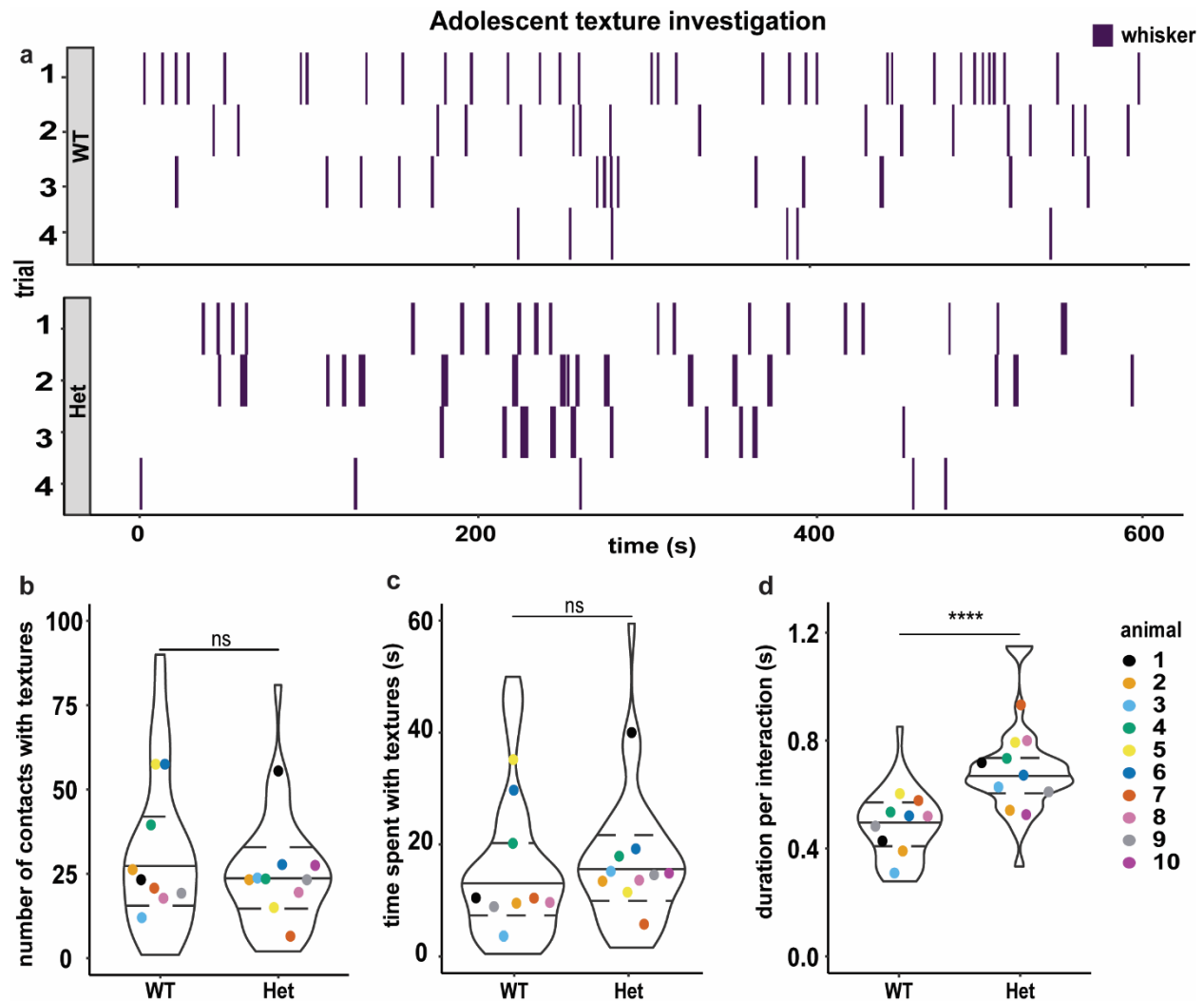


Figure 2.5. Adolescent Het have subtle whisking deficits during texture interactions.

(a) Examples of frame-by-frame texture interactions across four 10-min trials of texture exposure for adolescent WT (top panel) and Het (bottom panel), represented as sequences. The X-axis represents the time (s). Whisker interactions (purple) are plotted as blocks that span the duration of the interaction. Each row depicts fleeting whisker interactions of a mouse during individual trials. **(b-d)** Statistical analysis of the interaction quantifications revealed that on average, WT and Het had similar total number of contacts across 2-4 trials **(b)** and spent similar averaged total time investigating textures across 2-4 trials **(c)**. However, Het spent significantly longer during each whisker interaction than WT **(d)**. Superplots data visualization shows individual cohorts by same color circles, represent the mean of 2-4 trials (n=9 WT, n=10 Het) and violin plots show median and distribution of all interactions. Mann-Whitney test: ns = not significant, ****p < 0.0001.

Figure 2.6. Adolescent Het exhibit subtle whisking deficits during object interactions.

(a) Examples of frame-by-frame texture interactions across two 10-min trials of object exposure for adolescent WT (top panel) and Het (bottom panel) color coded by types of interaction (whisker, forepaw, whole-body climbing), represented as sequences. The X-axis represents the time (s), and interactions are plotted as blocks that span the duration of the interaction. Each row depicts fleeting whisker (purple), forepaw (yellow) or whole-body climbing (teal) interactions of the mice during individual trials. (b-d) Statistical analysis of the interaction quantifications revealed Het made significantly more contacts using whiskers (b), but not forepaws (c) or whole-body climbing (d) compared to WT. (e-g) There were no statistical differences between WT and Het for the time spent with objects using whiskers (e), forepaws (f), or whole-body climbing (g). (h-j) Het spent significantly more time than WT during each whisker interaction (h), but not with forepaw (i) or whole-body climbing (j). (b-j) Superplots data visualization shows individual cohorts by same color circles, represent the mean of 2 trials (n=9 WT, n=10 Het) and violin plots show median and distribution of all interactions. Mann-Whitney test: ns = not significant, **p < 0.01, ****p < 0.0001.

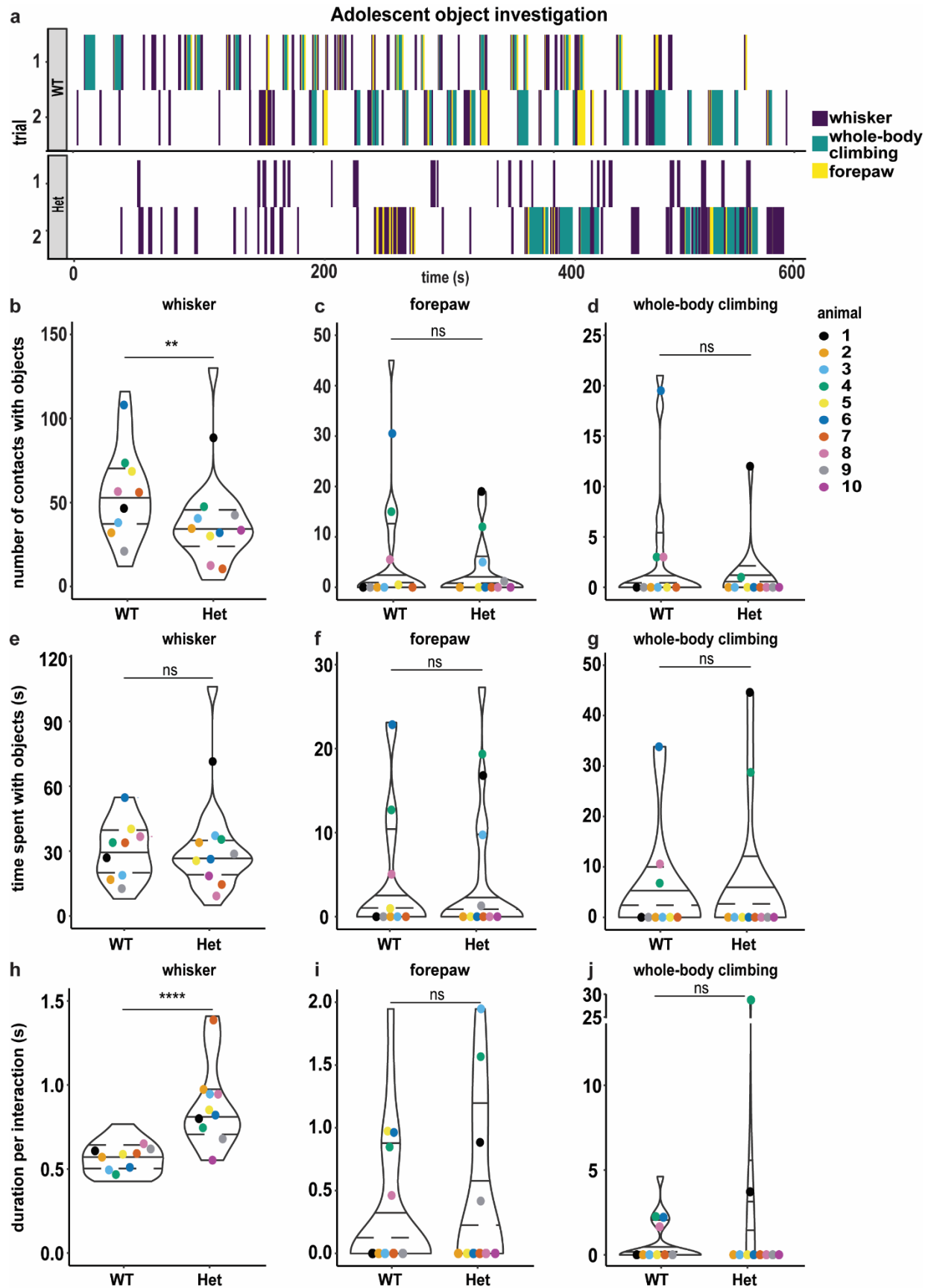


Figure 2.7. Adult Het exhibit hypersensitivity and compensatory behaviors during texture interactions.

(a) Examples of frame-by-frame texture interactions across four 10-min trials of texture exposure for adult WT (top panel) and Het (bottom panel) color coded by types of interaction (whisker, forepaw, whole-body climbing), represented as sequences. The X-axis represents the time (s), and interactions are plotted as blocks that span the duration of the interaction. Each row depicts fleeting whisker (purple), forepaw (yellow) or whole-body climbing (teal) interactions of the mice during individual trials. **(b-j)** Statistical analysis of the interaction quantifications revealed adult Het made significantly more contacts **(b-d)**, spent significantly more total time **(e-g)** and time per interaction **(h-j)** with textures, with all interaction types **(b-j)**, compared to adult WT. Superplots data visualization shows individual cohorts by same color circles,, represent the mean of 2-4 trials (n=9 WT, n=8 Het) and violin plots show median and distribution of all interactions. Mann-Whitney test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

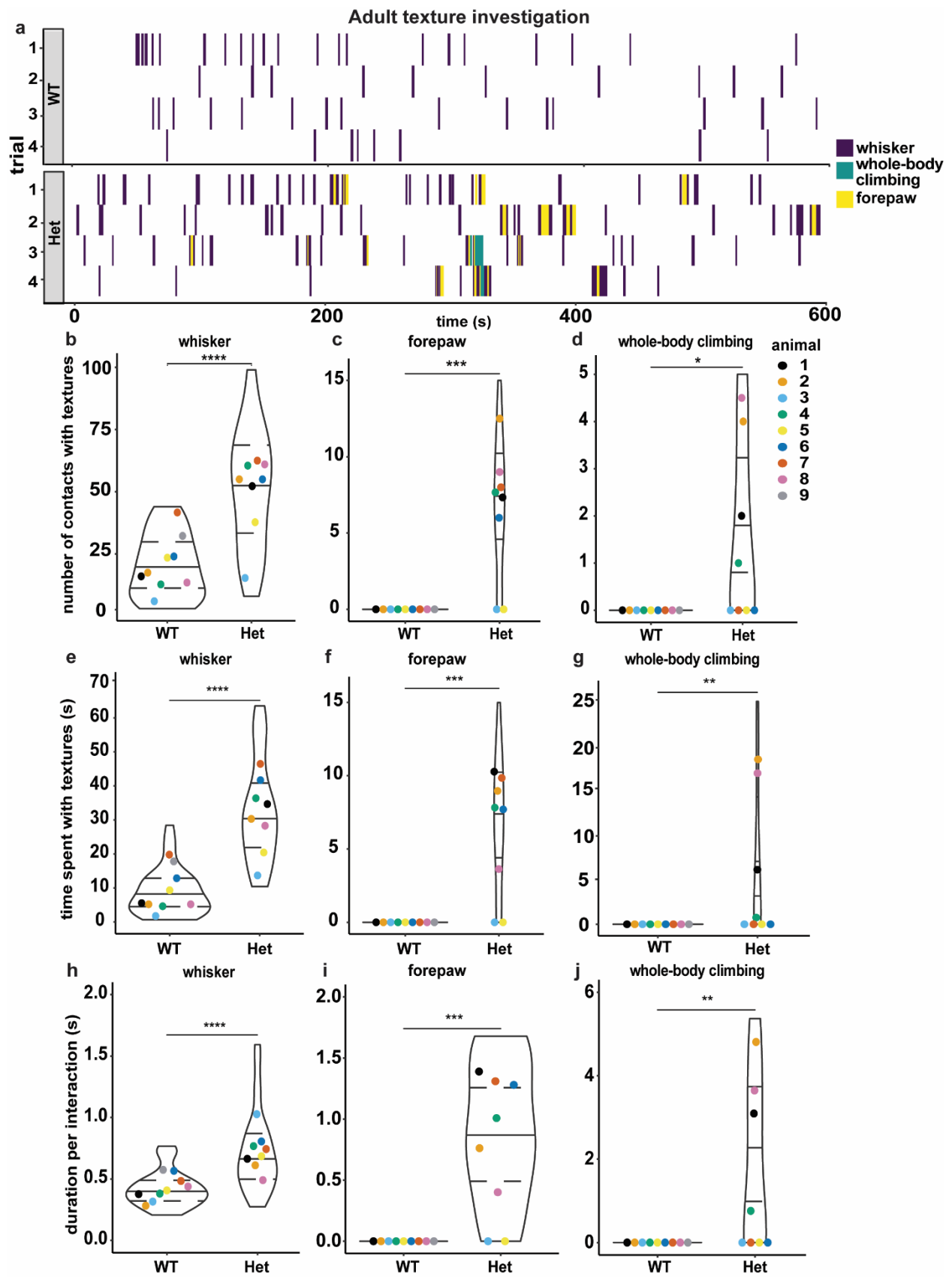
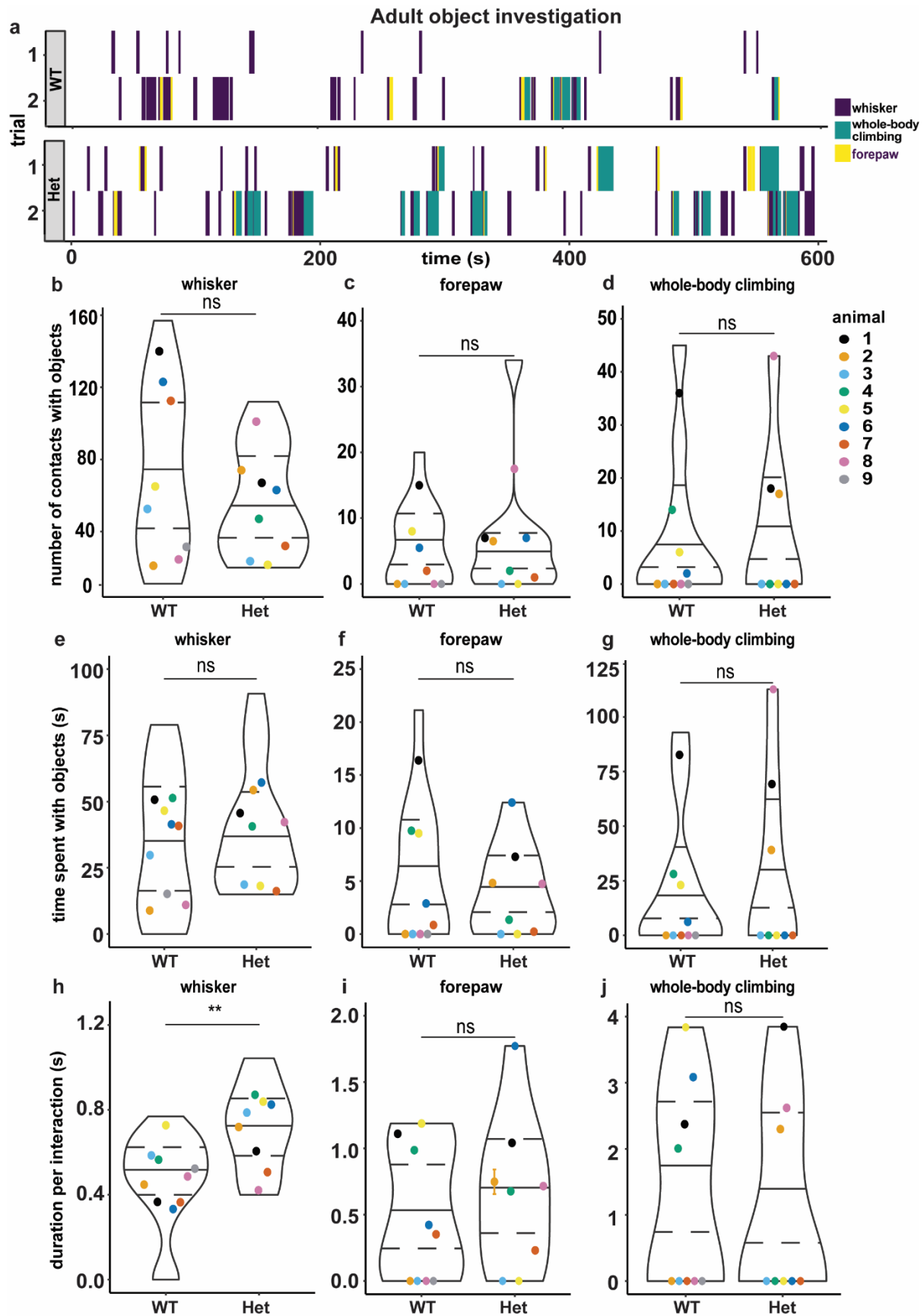


Figure 2.8. Adult Het display mild whisker deficit while interacting with objects. **(a)** Examples of frame-by-frame texture interactions across two 10-min trials of object exposure for adult WT (top panel) and Het (bottom panel) color coded by types of interaction (whisker, forepaw, whole-body climbing), represented as sequences. The X-axis represents the time (s), and interactions are plotted as blocks that span the duration of the interaction. Each row depicts fleeting whisker (purple), forepaw (yellow) or whole-body climbing (teal) interactions of the mice during individual trials. **(b-j)** Statistical analysis of the interaction quantifications revealed adult Het interacted with objects using whisker, forepaw and whole-body climbing, similar to WT, in the number of contacts **(b-d)**, total time with objects **(e-g)** and time per interactions (with Het having a slight but significant increase with whisker) **(h-j)**. Superplots show animals color coded by circles, representing the mean of 2 trials (n=9 WT, n=8 Het). Superimposition of violin plots show median and distribution of all interactions. Statistical significance determined by Mann-Whitney test: **p < 0.01.



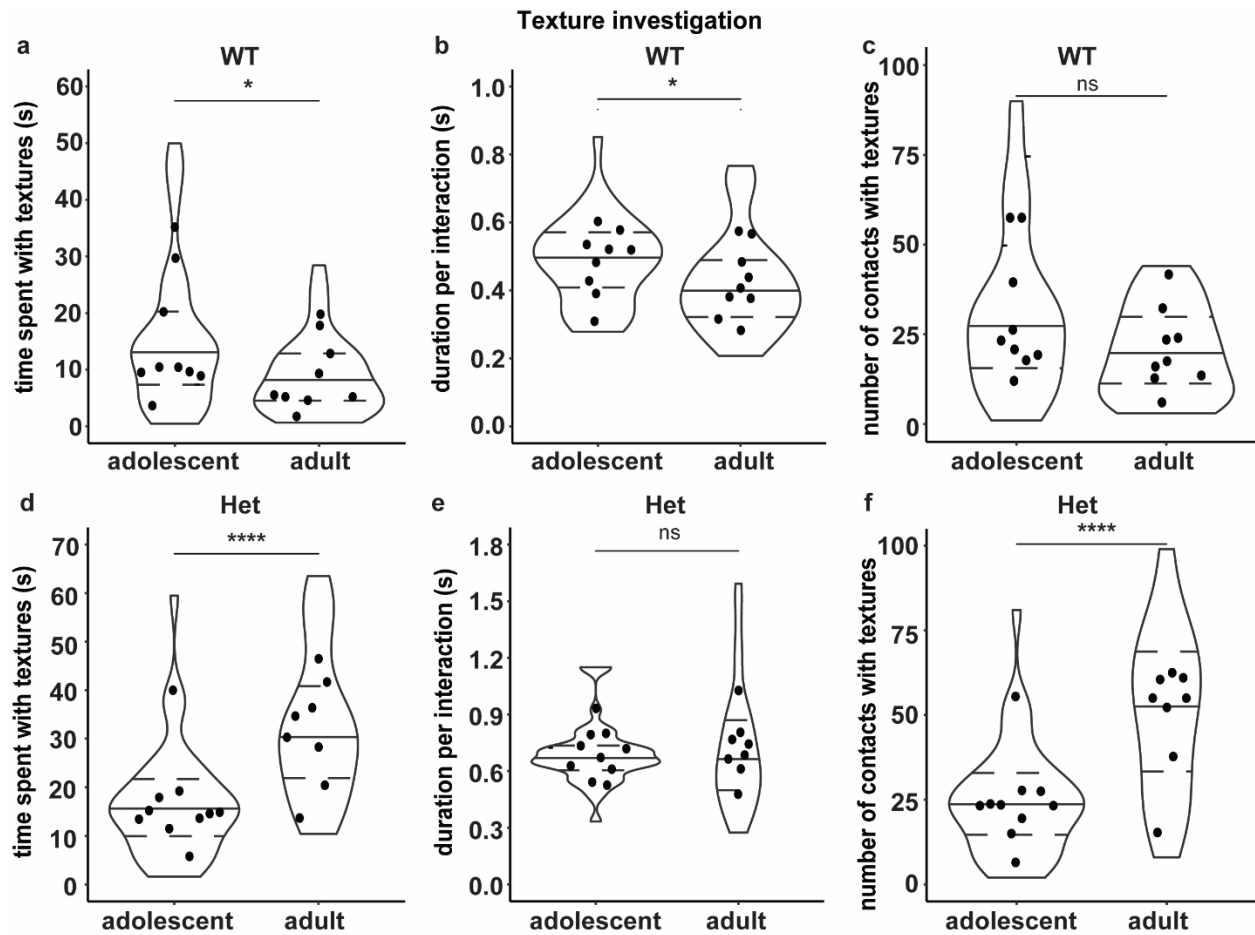


Figure 2.9. Differential and age-specific changes in WT and Het during texture interaction.

(a-c) Using whiskers, adult WT interacted in significantly shorter time (**a**: total time, **b**: time per interaction) with textures than adolescent WT, without changes in number of contacts made (**c**). **(d-f)** However, adults Het used whiskers to spent significantly longer time (**d**) and made more contacts (**f**) with textures than adolescent Het, without changes in time spent per interaction (**e**). **(a-f)** Each circle represents the mean of 2-4 trials per animal (adolescent: $n=9$ WT, $n=10$ Het; adult: $n=9$ WT, $n=8$ Het). Superimposition of violin plots show median and distribution of all interactions. Mann-Whitney test: ns = not significant, $*p < 0.05$, $****p < 0.0001$.

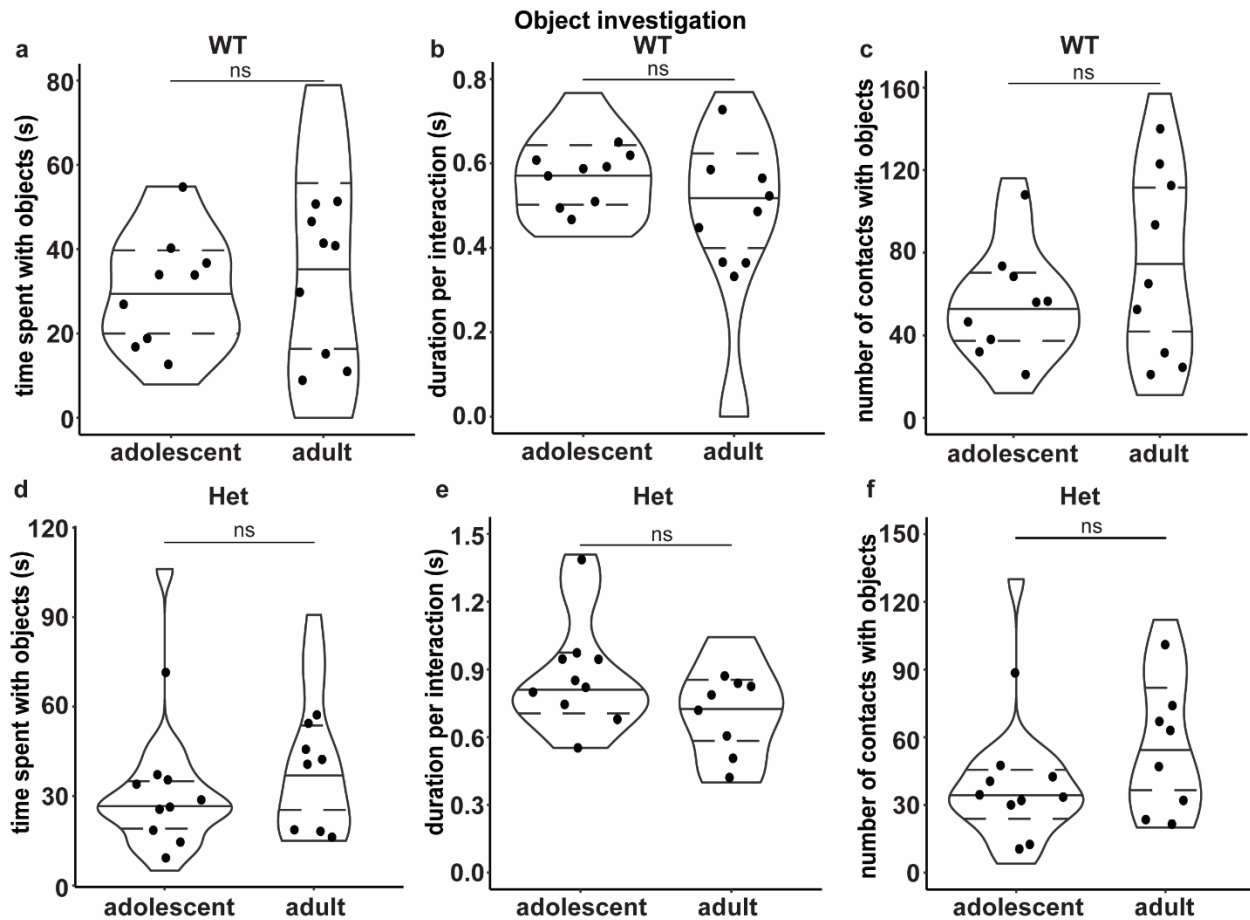


Figure 2.10. No differential or age-specific changes in WT and Het during object interaction.

(a-c) Both adolescent and adult WT used whiskers similarly to interact with objects, as determined by total time spent **(a)**, time per interaction **(b)** and number of contacts **(c)**. **(d-f)** Comparably, adolescent, and adult Het used whiskers similarly to interact with objects (**d**: total time; **e**: time per interaction; **f**: number of contacts). Each circle represents the mean of 2 trials per animal (adolescent: n=9 WT, n=10 Het; adult: n=9 WT, n=8 Het). Superimposition of violin plots show median and distribution of interactions. Mann-Whitney test: ns = not significant.

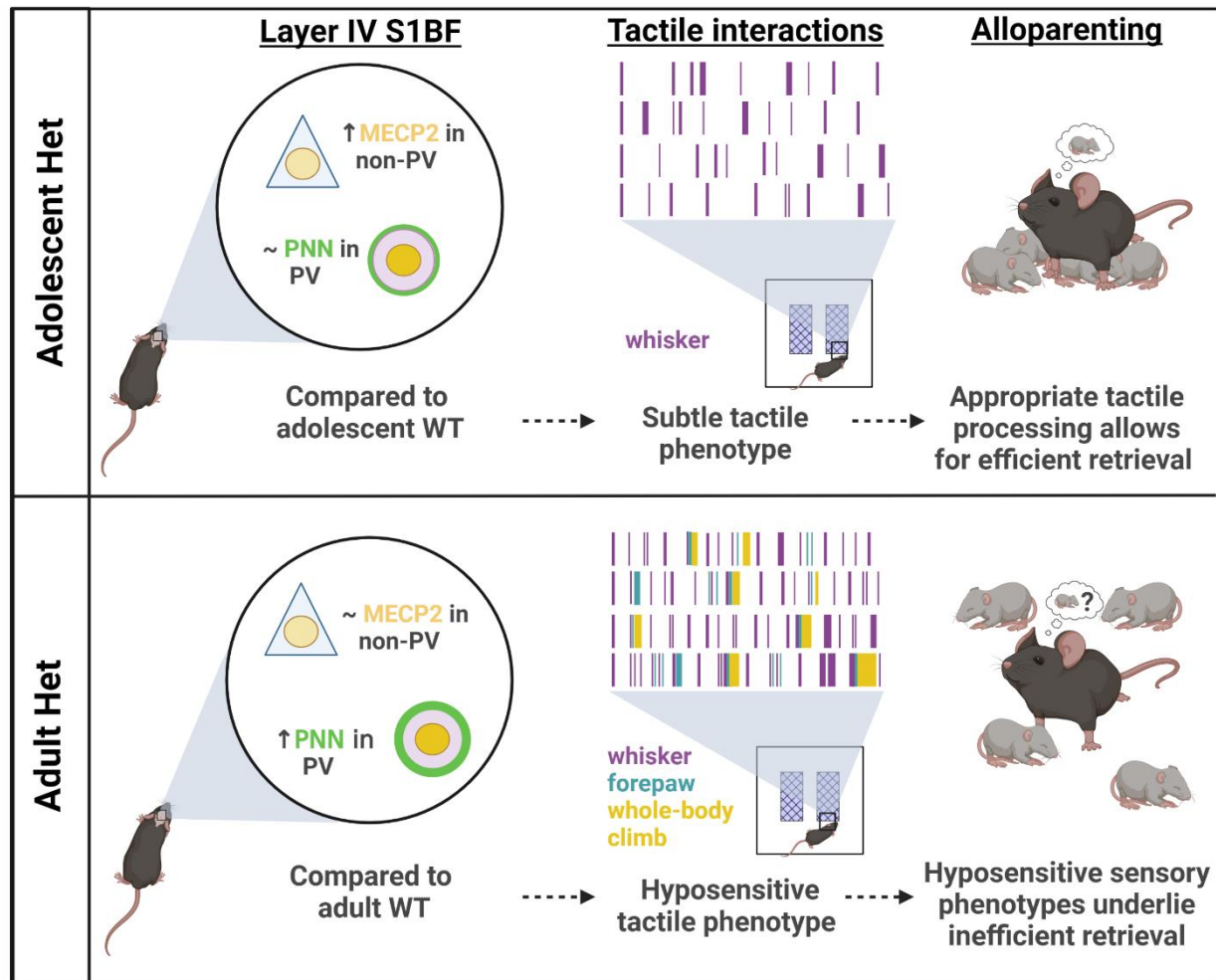


Figure 2.11. Summary diagram: MECP2 and PNN expression coincide with age-dependent sensory deficits in Het.

Wild-type MECP2 expression is dysregulated in genotypic adolescent *Mecp2*-heterozygous female mice, which coincides with typical expression of perineuronal nets in barrel field of the primary somatosensory cortex, mild tactile sensory perception, and efficient multisensory processing during the pup retrieval task. In adulthood, wild-type MECP2 expression in Het is similar to control WT brains, which coincides with atypical abnormal increase in perineuronal nets, strong whisker interactions indicative of a hyposensitivity phenotype, and aberrant multisensory processing leading to inefficient pup retrieval.

**CHAPTER 3: MULTIDIMENSIONAL ANALYSIS OF A SOCIAL BEHAVIOR
IDENTIFIES REGRESSION AND PHENOTYPIC HETEROGENEITY IN A FEMALE
MOUSE MODEL FOR RETT SYNDROME**

This work is under review at the Journal of Neuroscience: Link to Biorxiv <https://doi.org/10.1101/2023.06.05.543804>.

Abstract

Regression is a key feature of neurodevelopmental disorders such as Autism Spectrum Disorder, Fragile X Syndrome and Rett syndrome (RTT). RTT is caused by mutations in the X-linked gene Methyl CpG-Binding Protein 2 (MECP2). It is characterized by an early period of typical development with subsequent regression of previously acquired motor and speech skills in girls. The syndromic phenotypes are individualistic and dynamic over time. Thus far, it has been difficult to capture these dynamics and syndromic heterogeneity in the preclinical *Mecp2*-heterozygous (Het) female mouse model. The emergence of computational neuroethology tools allow for robust analysis of complex and dynamic behaviors to model endophenotypes in pre-clinical models. Towards this first step, we utilized DeepLabCut, a marker-less pose estimation software to quantify trajectory kinematics, and multidimensional analysis to characterize behavioral heterogeneity in Het over trials in the previously benchmarked, ethologically relevant social cognition task of pup retrieval. We report the identification of two distinct phenotypes of adult Het: Het that display a delay in efficiency in early days and then improve over days like wild-type mice, and Het that regress and perform worse in later days. Furthermore, regression is dependent on age, behavioral context, and is identifiable in early days of retrieval. Together, the novel identification of two populations of Het suggest differential effects on neural circuitry and opens new directions of exploration to investigate the underlying molecular and cellular mechanisms, and better design experimental therapeutics.

Introduction

Regression is defined as a loss of previously acquired motor skills over time and is a behavioral hallmark of neurodevelopmental disorders (Charman et al., 2002; Goin-Kochel et al., 2014; McVicar & Shinnar, 2004; Thurm et al., 2018). Despite decades of clinical observations that regression is prevalent in many neurodevelopmental disorders and that the occurrence of regression is higher than previously reported, the underlying cause of regression in most neurodevelopmental disorders remains elusive (Goin-Kochel et al., 2014; Goldberg et al., 2008; Ozonoff et al., 2018; Ozonoff & Iosif, 2019). Genetic mutations in proteins important for transcriptional regulation and experience-dependent synaptic plasticity in the brain are associated with higher risk for regression (Goin-Kochel et al., 2017; Tammimies, 2019). Amongst these targets is the X-linked gene Methyl-CpG-Binding-Protein 2 (MECP2), the monogenic cause for Rett syndrome (RTT), and a “hotspot” gene associated with other disorders such as Severe Neonatal-Onset Encephalopathy, PPM-X syndrome and microcephaly (Amir et al., 1999; Gonzales & LaSalle, 2010; Lambert et al., 2016; Rett, 1966; Schüle et al., 2008).

Most surviving RTT patients are girls and women who are heterozygous for *MECP2* mutations (Kirby et al., 2010). Female patients typically survive into their middle age, and exhibit sensory processing, cognitive and motor deficits throughout life (Buchanan et al., 2019; Djukic et al., 2012; Djukic & Valicenti McDermott, 2012; Key et al., 2019; LeBlanc et al., 2015; Merbler et al., 2020; Neul et al., 2010; Nomura, 2005; Peters et al., 2015; Stallworth et al., 2019; Symons et al., 2019). In these females, random X-chromosome inactivation leads to mosaic wild-type MECP2 expression and consequently, a syndromic phenotype. Broadly, Rett syndrome is diagnosed from two types of clinical presentations: 1) girls present with a short period of typical development, followed by regression, and expression of stereotypic sensory, motor, speech and cognitive impairments; or 2) developmental delay and expression of stereotypic sensory, motor, speech and cognitive impairments (Charman et al., 2002; Cosentino et al., 2019; Djukic & Valicenti McDermott, 2012; Einspieler & Marschik, 2019; Hagberg et al., 1983; Han et al., 2012; Neul et al., 2010, 2023; Symons et al.,

2019). Regression typically occurs between 6-18 months of age, and as late as 4 – 8 years from individual case studies (Buchanan et al., 2019; Han et al., 2012). These prolonged timelines of vulnerability suggest that the increased cognitive load required during early development while mastering motor control through exploration and interacting with the social environment might reveal specific features over ages. Though RTT is diagnosed in early development, regression in specific skills or learning is also observed throughout life. Currently, the underlying neural pathways that manifest in developmental delay, or regression after typical development and throughout different phases of life are unknown.

Preclinical *Mecp2* rodent models recapitulate many of the phenotypic features of RTT such as sensory processing, social communication, and motor deficits (Durand et al., 2012; Goffin et al., 2012; Ito-Ishida et al., 2015; Krishnan et al., 2017; Lau, Krishnan, et al., 2020; Lee et al., 2017; Lo et al., 2016; Mykins et al., 2023; Orefice et al., 2016; Stevenson et al., 2021; Su et al., 2015; Veeraragavan et al., 2016; Xu et al., 2022). Thus far, three other published papers have shown possible regression phenotypes, for breathing abnormalities in a mouse conditional knockout model, learned forepaw skill involving seed opening in a female rat model, and in skilled motor learning in female mice (Achilly, Wang, et al., 2021; T.-W. Huang et al., 2016; Veeraragavan et al., 2016). In these models, deterioration in learned skills was noted over multiple weeks. However, reliably identifying regression within a social behavioral task in apt preclinical models, with shorter time resolution in order to measure impact of therapeutics, has remained a challenge.

Towards this end, we have established an ethologically relevant social behavioral assay called pup retrieval task as a model to study cellular and neural circuitry basis for social cognition, complex sensorimotor integration and experience-dependent plasticity (Dvorkin & Shea, 2022; Krishnan et al., 2015, 2017; Lau, Krishnan, et al., 2020; Lau, Layo, et al., 2020; Mykins et al., 2023; Stevenson et al., 2021; Xie et al., 2023). During this task, female mice integrate sensory cues to execute goal-directed motor sequences to retrieve scattered pups back to their home nest (Alsina-Llanes et al., 2015; Beach &

Jaynes, 1956; Champagne et al., 2007; Champagne & Curley, 2016; Dunlap et al., 2020; Hemel, 1973; Koch & Ehret, 1989; Lonstein et al., 2015; Marlin et al., 2015; Stern, 1996; Stern & Mackinnon, 1978). Using end-point metrics such as latency index and errors during retrieval, we found that 6-week-old adolescent *Mecp2*-heterozygous female mice (Het) were as efficient at pup retrieval as wild-type littermate controls (WT), and 12-week-old adult Het were inefficient at retrieval (Krishnan et al., 2017; Mykins et al., 2023; Stevenson et al., 2021). However, movement sequences during social behaviors are dynamic over time, with distinct behavioral sequences that are often ignored due to their inherent complexity (Stevenson et al., 2021).

The emergence of computational neuroethology tools allows for fast, reliable, and systematic detection of recorded movement over time to extract multiple metrics in different contexts. This extraction is critical for identifying unique sub-populations and assessing the therapeutic value of manipulations for pre-clinical animal studies for psychiatric disorders (Luxem et al., 2023; Popovitz et al., 2021; Shemesh & Chen, 2023; Tanas et al., 2022; Yamamoto et al., 2018). Thus, to capture the complexity of individual variations in a mosaic *Mecp2*-heterozygous population over multiple days of pup retrieval behavior, we used DeepLabCut, a marker-less pose estimation software, to derive animal trajectories, and performed multidimensional analysis of trajectory kinematics in adolescent and adult female WT and Het (Mathis et al., 2018; Nath et al., 2019). The goal of this study was to determine if DeepLabCut can provide better behavioral characterization and aid in identifying regression in this heterogeneous population of Het.

Here, we report the identification of robust phenotypic variation and regression in genotypic adult Het. Particularly, we identified genotypic Het that had milder phenotypes, similar to adult WT in many features (Het non-regressors, Het-NR), and another distinct group of genotypic adult Het that exhibited severe inefficiency in consolidating the pup retrieval movements over days (Het regressors, Het-R). Interestingly, the regression was dependent on context as we observed WT, Het-NR and Het-R clustered together in Principal Component space during habituation and

isolation phases, suggesting Het-R have specific issues likely in integrating sensory and motor sequences during the decision/execution phase of retrieval. Additionally, this inefficiency in consolidation was specific to adulthood as adolescent Het behaved similarly to adolescent WT, indicating genotypic Het are able to perform this complex pup retrieval task at an earlier time point in life, and are not able to maintain goal-directed movement skills as they age. The novel identification of two populations of Het-NR and Het-R suggests differential effects on neural circuitry and opens new directions of exploration on cellular mechanisms for compensation and maladaptive plasticity. Together, these results emphasize the need to analyze dynamics in individual variations and context-dependency while considering therapeutic and treatment options.

Materials and Methods

Animals

We used the following mouse strains: CBA/CaJ (JAX:000654) and *Mecp2*^{heterozygous} (Het) (C57BL/6J, B6.129P2[©]-*Mecp2*^{tm1.1Bird}/J, JAX:003890) and wild-type littermate controls (WT) (Guy et al., 2001). All animals were group-housed by sex after weaning, raised on a 12/12-hour light/dark cycle (lights on at 7 a.m.) and received food and water ad libitum. Behavioral experiments were performed using 6-week-old (adolescent) and 10–12-week-old (adult) Het and WT between the hours of 9 a.m. and 6 p.m. (during the light cycle). All procedures were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Tennessee-Knoxville.

Pup Retrieval Task

Behavioral Paradigm

The pup retrieval task was performed as previously described (Mykins et al., 2023; Stevenson et al., 2021). Two 6-week-old or 10–12-week-old naïve female littermates (one WT and one Het) with no prior experience of pup retrieval were co-housed with a pregnant CBA/CaJ female (10-12 weeks of age) 3-5 days before birth. Once the pups were born (Postnatal Day 0; D0), we performed the pup retrieval task once a day for six consecutive days, in the home cage which was placed inside of a sound- and light-proof

box. The behavioral task was performed as follows (**Figure 3.1.a**): one mouse was placed in the home cage with 3-5 pups (Habituation; 5 minutes), the pups were removed from the nest (Isolation; 2 minutes), and the pups were scattered by placing them in the corners and center, allowing the mouse to retrieve pups back to the nest (Retrieval; 5 minutes at max). The nest was left empty if there were fewer than five pups. The assay was performed in the dark and recorded using an infrared camera (Foscam Wired IP Camera) during all phases of each trial. If all pups were not retrieved to the nest within 5 minutes, we removed the mouse and placed pups back in the nest in preparation for the next mouse. While one of the females was performing the pup retrieval task, the mother, her pups, and the other adult were housed in a separate new cage. After the behavior was completed, all mice and pups were returned to the home cage.

Behavior Analysis:

All recorded videos were coded so the analyzers were blind to the identity of the mice. Each video was manually scored using a latency index (the amount of time to retrieve all pups back to the nest out of 5 minutes) and the number of errors that represent adult-pup interactions that did not result in successful retrieval).

Latency index was calculated as: $Latency\ index = [(t_1 - t_0) + (t_2 - t_0) + \dots + (t_n - t_0)] / (n \times L)$

Where n = number of pups outside the nest, t_0 = start time of trial (s), t_n = time of the nth pup's successful retrieval to the nest (s), L = total trial length (300 s). Statistical analyses and figures were generated using R studio.

Pose estimation generated from DeepLabCut:

For body part tracking, we used DeepLabCut (version 2.2.0.3) (**Figure 3.1.b**). We defined features of interest as the mouse's nose, left ear, right ear, shoulder, spine 1, spine 2, left hind limb, right hind limb, and tail base. We labeled 200 frames of features of interest taken from 25 videos of WT and Het during pup retrieval phase and then 95% was used for training. We used a ResNet-50-based neural network with default parameters and trained for 720,000 iterations on Google Colab (Insafutdinov et al., 2016). We performed outlier correction to correct inaccurate predictions from the

DeepLabCut model. In the Graphical User Interface (GUI), we refined 50 frames of predicted labels by moving the predicted label's location to the actual position of the body part of interest (**Figure 3.1.b**). We then retrained, validated with 1 number of shuffles, and found the test error of was 1.67 pixels, and train error was 1.66 pixels (image size was 640 X 480 pixels). We then used a p-cutoff of 0.9 to condition the X and Y coordinates for future analysis. This model network was then used to analyze novel behavioral videos of adult animals. We then repeated this process for 6-weeks-old adolescent pup retrieval behavior. We labeled 200 frames taken from 20 videos of 6-weeks-old pup retrieval behavior, trained for 1,030,000 iterations, refined 75 frames of predicted labels, retrained, and validated with 1 number of shuffles, and found the test error of was 1.67 pixels, and train error was 2.85 pixels.

Pose estimation derived trajectory analysis:

To quantify behavioral trajectories during habituation, isolation and retrieval, we used the Traj R package (McLean & Skowron Volponi, 2018) (**Figure 3.1.c**). We filtered the X and Y coordinates of the nose from the DLC pose and quantified 13 Trajectory metrics (11 metrics were used for habituation and isolation as they did not have latency index and error) (**Table 2.1**). For habituation and isolation phases, we analyzed trajectories during the entire 5-minute and 2-minute trial phases, respectively. For pup retrieval phase, we analyzed trajectories during the act of retrieval; we stopped analyzing trajectories after all pups have been retrieved.

Multidimensional analysis of pup retrieval trajectories:

For multidimensional analysis of behavior profiles, we performed data selection, standardization, principal component analysis (PCA), k-means clustering, and validation (Fig. 2b) (Popovitz et al., 2021; Tanas et al., 2022). We utilized PUMBAA (phenotyping using a multidimensional behavioral analysis algorithm, <https://github.com/sidorovlab/PUMBAA>) for all multidimensional analysis (Tanas et al., 2022).

Data selection for retrieval:

For individual and combined-days analyses of retrieval, 13 X number of days of metrics were included in multidimensional analysis or PCA analysis.

Data selection for habituation and isolation:

For all days' analysis, a total of 66 metrics (11 metrics across 6 days) were included in multidimensional analysis (**Figure 3.4.b, e**). For combined D1 and D5 analysis, 22 metrics were included in multidimensional analysis (**Figure 3.4.c-f**).

Standardization:

All measures were standardized using z-score normalization ($z = (\text{data point} - \text{group mean}) / \text{standard deviation}$). This accounts for different unit magnitudes across metrics similar to previous studies (Popovitz et al., 2021; Tanas et al., 2022).

Principal component analysis and K-means clustering:

We performed PCA using PUMBAA and extracted the amount of variance explained by each principal component (**Figure 3.3.b**). We performed k-means clustering in principal component space using PUMBAA with $k = 2$ clusters in 2 Principal Component (2PC) space. To verify visual absence of clusters, we performed a gap statistics test using the cluster package in R. Data visualization using ggplot were performed in R studio (Wickham, 2016).

Validation:

We compared the genotypes to their predicted k-means cluster. We color-coded groups according to their predictions (WT predicted correctly= black, Het predicted correctly=red, and Het predicted incorrectly=light blue) (**Figure 3.2**). This allowed us to determine the accuracy of predicting genotypes in our model and revealed two distinct populations with the same genotype. Data visualization using ggplot was performed in R studio (Wickham, 2016).

Using predicted clusters to analyze behavior:

Using the identified clusters of Het from **Figure 3.2.f**, we performed trajectory analysis for metrics contributing significantly to the PCA. We conducted the Kruskal-Wallis test

with uncorrected Dunn's test to determine the statistical significance of metrics of interest between WT and the two clusters of Het (**Figure 3.3**) on each day and within the same group across days. We used ggplot and the dunn.test package to perform statistical analysis and data visualization in R studio (Wickham, 2016).

Results

Robust identification of phenotypic heterogeneity in adult Het during pup retrieval:

Previously, we reported that the adult female Het were inefficient at pup retrieval, as measured by time taken to retrieve pups back to the nest, and increased physical interactions between pups and adult that did not result in efficient retrieval (Krishnan et al., 2017; Stevenson et al., 2021). We also reported individual variations with possible regression phenotype over days in adult Het, from frame-by-frame manual analysis of goal-directed movements during pup retrieval in a small sample size (N=6 females per genotype) (Stevenson et al., 2021). These intriguing results identified the need for systematic, automated and unbiased analysis for large sample sizes over multiple days. Additionally, we sought to identify newer dynamic metrics in context-specific motor sequences, in addition to the established end-point measurements.

To achieve these goals, here we used DeepLabCut to generate animal pose and quantified behavioral trajectory profiles across pup retrieval days in adolescent (N=9, WT and N=11, Het) and adult female WT and Het (N=21 per genotype) (Mathis et al., 2018) (**Figure 3.1.a, b**). We incorporated animal trajectory analysis that included 13 metrics (Table 1), and adapted multidimensional analysis methods to cluster animal phenotypes, based on their genotypes and behavioral profiles (**Figure 3.1.c**) (Benhamou, 2004, 2014; Bovet & Benhamou, 1988; Cheung et al., 2007; McLean & Skowron Volponi, 2018; Tanas et al., 2022). Together, we took 13 metrics from each day of retrieval (13 metrics per day) or across 6 days of pup retrieval (78 metrics total) to build a behavioral profile for each mouse (**Figure 3.2.a**). We standardized all metrics using z-scores and performed multidimensional principal component analysis (PCA) (**Figure 3.2.a, Scaled PCA**) (Popovitz et al., 2021; Tanas et al., 2022). We then used k-

means to cluster mice into two groups based on genotype and validated clustering by comparing the predicted cluster group to the known genotype (WT vs Het) of the animal **(Figure 3.2.a, K-means and Validation, respectively)**. Based on this analysis, we identified a distinct group of Het clustered at the top of principal component (PC) space (Het-R, red) while another cluster with WT (Het-NR, blue) **(Figure 3.2.b)**. When we performed multidimensional PCA analysis on each day of retrieval, we observed the Het-R cluster emerged as early as D1, however, it consolidated with WT and other Het on D2 and D3 and emerged again on D4 and D5 **(Figure 3.2.c)**. This suggests heterogeneity in phenotype is dynamic over days. Pairwise combinations between D0:D5, D1:D5, D2:D5, D3:D5 and D4:D5 **(Figure 3.7)** indicated multidimensional analysis of D1 and D5 is sufficient to identify the two clusters of Het via PCA, k-means and validation **(Figure 3.2.d-f)**. Furthermore, PC1 alone distinguished the separation between the two clusters of Het **(Figure 3.2.g)**. Together, these novel results robustly quantify the known behavioral heterogeneity in adult Het over multiple days and identify regression in a subpopulation of adult Het.

Identification of trajectory metrics that classify behavioral heterogeneity over days:

In order to better characterize the three phenotypes (WT, Het-NR, Het-R), we plotted the trajectories, and observed striking differences in the movement patterns over days **(Figure 3.3.a)**. Similar to WT, Het-NR trajectories were random on D0 and consolidated to oriented and direct paths toward the nest by D5 **(Figure 3.3.a)**. Het-R also had random trajectories on D0 but showed instability in consolidating trajectories over time compared to WT and Het-NR **(Figure 3.3.a)**.

To determine the identity of the most significant variables, we plotted the variable vectors in principal component (PC) space **(Figure 3.3.b)** for D1 & D5, as this allowed us to isolate the regressors with the least number of dimensions **(Figure 3.2.f)**. Sinuosity expected maximum displacement (emax) and expected maximum displacement B (emaxB) contributed to PC1, and distance, duration, nonlinearity, irregularity, latency index, and error contributed to PC2, whereas mean acceleration and

straightness had negative correlations and did not contribute to either principal component (**Figure 3.3.b**). Positive correlations between emax, emaxB, mean speed, mean step length, and negative correlation with sinuosity contributed to PC1 & PC2 (**Figure 3.3.c**). Breakdown of contributions by PC1 (**Figure 3.3.c**) and PC2 (**Figure 3.3.d**) indicated nonlinearity, irregularity, distance, and latency contributed to the most variability in PC2.

We previously reported latency index, or the time it takes to retrieve all pups, as an endpoint to evaluate pup retrieval performance (Krishnan et al., 2017; Mykins et al., 2023; Stevenson et al., 2021). As a positive control, we also quantified latency index for this larger dataset. As expected, WT and Het-NR significantly decreased in latency index over days compared to baseline, here defined as D0 performance, within genotype (**Figure 3.3.e, e', WT, Het-NR**). This suggests learning and consolidation towards efficient pup retrieval task over days. However, Het-NR were significantly worse than WT on D1, D2, D4 and D5 (**Figure 3.3.e, e', WT vs Het-NR**), suggesting the possibility of intact learning and consolidation mechanisms, albeit not as well as WT controls. Similar to WT and Het-NR trends, Het-R latency index significantly decreased in early days (**Figure 3.3.e, e', Het-R**). However, Het-R latency increased from D4 to D5 and reverted to D0 levels. Het-R were significantly worse than WT on all days (**Figure 3.3.e, e', WT vs Het-R**) and were significantly worse than Het-NR on D5 (**Figure 3.3.e, e', Het-NR vs Het-R**). Together, these results suggest Het-R display delay in learning efficiency and are unable to consolidate efficient patterns over time.

Features of Het regression are context-dependent

Given the dynamic changes in trajectory metrics during pup retrieval over days in the Het-R, we wondered if these features were specific to the act of pup retrieval, or other generalized motor features in this subpopulation. Thus, we performed principal component analysis of trajectories during the habituation and isolation phases of behavior (**Figure 3.4**). As latency and error metrics are specific to adult and pup interactions during retrieval, we excluded them in the multidimensional analysis. During habituation, the pups are grouped together in the nest and the adult can choose to

interact/huddle with pups or not (**Figure 3.1.a**). This serves as a control to determine if the context and action of retrieving pups distinguishes Het-NR and Het-R. We plotted the habituation trajectories (example plots for WT (black), Het-NR (blue) and Het-R (red)) and observed no differences in the patterns of their trajectories between D0 and D5 (**Figure 3.4.a**). Both genotypes tend to move around the entire cage during the five-minute habituation phase. No distinct groups emerged between WT, Het-NR and Het-R from the differential PCA analyses (**Figure 3.4.b, c**).

During the isolation phase, the pups are removed from the home cage, and the adult is left alone for two minutes (**Figure 3.1.a**). We plotted the isolation trajectories of the three groups and observed no differences in the patterns of their trajectories between D0 and D5 (**Figure 3.4.d**). Similar to habituation phase, both genotypes moved about the cage with no pups present, and no distinct groups emerged from the isolation specific PCA analyses (**Figure 3.4.e, f**). These findings are consistent with the manual frame-by-frame analysis of maternal behaviors, which demonstrated WT and Het behave similarly during habituation and isolation (Stevenson et al., 2021). Together, these results suggest context-specific, atypical behavioral features of Het-R and Het-NR, and not generalized motor phenotype in genotypic adult Het. This is a critical finding which points to context-specific processing of sensorimotor information in genotypic Het and subsequent execution of specific motor sequences in a social context.

Analysis of early days identifies individual variation in later adult Het regressors.

In order to determine individual variations in Het, we plotted individual trajectories of Het-NR and Het-R over all days. As expected, we observed that Het-NR, such as animals 8, 20 and 21, displayed some variability in adapting over time, compared to the other Het-NR animals that plateaued and consolidated their efficiency in later days (**Figure 3.5.a**). In contrast, we observed variability in individual trends in Het-R. Animal 10 did not improve in pup retrieval efficiency over days, animals 3, 4, 6, 9, 19 improved over days but regressed by D5 and animal 2 improved over days but showed instability

in D4 (**Figure 3.5.a'**), suggestive of different neural mechanisms being disrupted in individual Het over days.

While it has been difficult to phenotype regression in Het mouse models of RTT, it is an even greater challenge to determine the time, frequency and duration to apply therapeutic intervention to mitigate regression before its onset. We thus asked if it was possible to identify the Het-R population (**Figure 3.2; Figure 3.5.a'**) from the initial days of pup retrieval performance. Using D0 & D1 metrics (**Figure 3.5.b**), we obtained weak informative power for identifying majority regressors, with 43% accuracy. However, combinations of D0-D2 (**Figure 3.5.c**) and D0-D3 (**Figure 3.5.d**) metrics elicited stronger informative power for identifying majority of regressors early on, with 71% accuracy. Despite observations from select metrics that show Het-R trends like WT and Het-NR in early days (**Figure 3.3.e, f, g**), regression in social behavior can still be identified using all metrics (**Figure 3.5.c, d**). Together these results suggest that we can identify potential regressors with a smaller number of trials which has implications for designing preclinical therapeutic studies in the future.

Experience dependent regression in Het is specific to age

We previously reported 6-week-old (adolescent) Het perform pup retrieval comparable to adolescent WT, using end-point metrics of latency index and error (Mykins et al., 2023). This is a particularly important result that shows that genotypic Het can perform as efficiently as the WT at an earlier age, which then results in regression features in adulthood. To determine if dynamic pose estimation metrics can identify early signs of regression in adolescent Het, we generated another deep learning model and pose for adolescent WT and Het animals, and quantified trajectory metrics. We did not observe any distinct genotype groups through PCA analysis (**Figure 3.6.a, b**). In support of this finding, we performed the gap statistic test and determined that the optimal number of clusters between both genotypes was one (**Figure 3.6.a', b'**).

To determine if there are developmental differences in retrieval trajectories between adolescence and adulthood within each genotype, we performed PCA on all metrics for

all days (**Figure 3.6.c**) and D1 & D5 (**Figure 3.6.d**) between adolescent and adult WT. We observed no distinct groups between adolescent WT (grey) and adult WT (black) (**Figure 3.6.c, d**). We performed PCA on 13 metrics for all days (**Figure 3.6.e**) and D1 & D5 (**Figure 3.6.f**) between adolescent and adult Het. In all day analysis, the majority of adolescent Het grouped with adult Het-NR (**Figure 3.6.e**), however, one adolescent Het grouped with adult Het-R. Interestingly, in D1 & D5 only, adolescent Het were mixed with adult Het-NR and adult Het-R (**Figure 3.6.f**). Together, these results suggest possible identification of adolescent Het at risk for regression over time. Future large scale and longitudinal studies are needed to address these possibilities.

Discussion

One of the major hurdles in diagnosing, understanding, and treating heterogeneous rare neuropsychiatric diseases is the lack of appropriate tools to robustly identify and classify complex morbidity over time and trials with individual resolution. While males are typically severely affected by MECP2 dosage, females typically exhibit less severe and more heterogeneous phenotypes due to random X-chromosome inactivation and mosaic expression of the wild-type protein. Thus, disease pathobiology caused by X-linked mutations, and by MECP2, is fundamentally different in females than males. Currently, there is minimal consensus on behavioral phenotypes in female Het mice, due to strain variations and lack of systematic analysis over different ages (Fagiolini et al., 2020; Garg et al., 2013; Krishnan et al., 2017; Mykins et al., 2023; Ribeiro & MacDonald, 2020; Samaco et al., 2013; Stearns et al., 2007; Stevenson et al., 2021). This is particularly important for designing better therapeutic interventions, as symptoms change over time in individual patients. Thus, developing and utilizing appropriate preclinical models are a necessary step. Here, we utilize pose estimation algorithm and multidimensional analysis to characterize complex social cognition phenotypes in the heterogeneous population of adolescent and adult female *Mecp2*-heterozygous mice. We found context- and age-specific regression in a subset of genotypic Het (Het-Regressors, Het-R) and an age-specific delay in efficient behavior in another subset of genotypic Het (Het-non regressors, Het-NR). These are novel results that finally achieve categorization of known, yet elusive, phenotypic heterogeneity in female X-linked

disorders. Additionally, this is the first report to faithfully recapitulate regression and mild developmental delay in the apt preclinical model. This robust phenotyping in individual animals will be critical in determining the mechanisms connecting the known MECP2 mosaicism, etiology and disease progression in Rett syndrome over time, contexts and physiological states. Future work will be needed to correlate heterogeneity in disease severity to known models of MECP2 mutations, and in different relevant behavioral models (Ehrhart et al., 2021; Neul et al., 2008; Vashi & Justice, 2019). The implications of such approaches will be in establishing robust and reproducible phenotyping of female mice across labs, provide biomarkers through longitudinal progression, physiological ages and symptom severity with individual resolution, for better design for clinical interventions (Leonard et al., 2022).

Pup retrieval task as ethologically relevant task to study complex social cognition:

Maternal behavior and pup retrieval tasks are well-established paradigms to study complex dyadic interactions focusing on neurological processes such as motivation, goal-directed movements, sensory processing and perception, social communication and cognition (Alsina-Llanes et al., 2015; Beach & Jaynes, 1956; Champagne et al., 2007; Champagne & Curley, 2016; Lonstein et al., 2015; Morgan et al., 1992; Rosenblatt, 1967; Stern, 1996; Stern & Kolunie, 1989; Stevenson et al., 2021; Wiesner & Sheard, 1933). In our assay, naïve virgin female mice are co-housed with a pregnant female, 3-5 days before birth of pups. Once pups are born, we remove pups from the nest and scatter them in the home cage, allowing for individuals to retrieve the pups back to the nest (pup retrieval behavior), over six consecutive days. This scenario is akin to a person moving to a new place with roommates, and then motivated (or induced) to help/interact and care for newborn babies, without any prior experience, for short periods of time every day. It is a challenging task to navigate this complex social environment, with demands on cognitive flexibility, sensory processing and perception, and skilled motor sequences. The purpose of using a complex social task, which taps into a combination of innate and learned neural circuitry, in this heterogeneous population is based on the premise that brains are evolved to engage and process

complex environmental and social information; thus, challenges will reveal dynamic and nuanced phenotypes in behaviors and neural processing over time and contexts, with reproducible variations in individual animals. Thus, such complexity is critical for better utilization of preclinical models of human disorders.

At the neurobiology level, much is known about the contributions of specific brain regions and neuromodulatory systems for efficient maternal care. Particularly, when nulliparous naïve female rodents are first exposed to pups, over several days, they adapt to retrieve pups, and co-housing with a pregnant female increases efficiency of pup retrieval (Cohen et al., 2011; Ehret et al., 1987; Hemel, 1973; Krishnan et al., 2017; Marlin et al., 2015; Stevenson et al., 2021). The neural circuit mechanism by which naïve female mice adapt repetitive and stereotyped motions over days to efficiently retrieve scattered pups back to the nest is still under investigation. Perturbation in dopaminergic mesolimbic pathway, ventral tegmental area and medial prefrontal cortex serotonergic neurons impair sequential pup retrieval and maternal behavior, suggesting a role for neuromodulation in motivation and reward of the pup (Gao et al., 2018, 2020; Hansen, 1994; Hansen et al., 1991a, 1991b, 1993; Keer & Stern, 1999; Wu et al., 2016; Xie et al., 2023). Dopaminergic activity increases in the ventral tegmental area and nucleus accumbens before pup contact and is required for reinforcement learning of pup retrieval over days in nulliparous female mice (Dai et al., 2022; Xie et al., 2023). Many of these established circuits have strong projections into higher cortical regions important for sensory motor integration. In this study, we have established metrics indicating naïve wild-type mice are adapting sensorimotor strategies to increase retrieval efficiency potentially to maximize the perceived social reward from pups. We hypothesize mice prioritize sensory-dependent social reward of the pups in early days of retrieval and utilize higher cortical regions to consolidate motor sequences in later days. Future work using *in-vivo* optogenetic manipulation combined with real-time pup retrieval dynamics to investigate the role of sensorimotor neural circuits is important to elucidate the underlying neural mechanisms of this relevant task.

Neurobiology underlying sensory and social communication deficits in Rett syndrome:

Longstanding work in the field of Rett syndrome has identified disruptions in cortical and subcortical regions to be major factors for sensory, motor, and social communication deficits in predominantly *Mecp2-null male* rodent models. Additionally, studies with cell-type specific deletions and “rescues” of *Mecp2* have identified particular contributions of cell-autonomous and non-cell autonomous effects in these phenotypes (Achilly, He, et al., 2021; Achilly, Wang, et al., 2021; Artoni et al., 2020; Ballinger et al., 2019; Bhattacharjee et al., 2017; Chao et al., 2010; Chapleau et al., 2009; Gemelli et al., 2006; Goffin et al., 2014; He et al., 2014; Ito-Ishida et al., 2015; Krishnan et al., 2017; Meng et al., 2016; Morello et al., 2018; Mossner et al., 2020; Orefice et al., 2016, 2019; Xu et al., 2022; Zhao et al., 2022). Specifically, mice with conditional deletion of *Mecp2* in parvalbumin+ GABAergic (PV) neurons develop a wide range of functional and behavioral RTT-like symptoms including atypical sensory-motor learning and pup retrieval behavior (Ito-Ishida et al., 2015; Krishnan et al., 2017; Mossner et al., 2020; Patrizi et al., 2020). An altered PV-network configuration interferes with experience-dependent plasticity mechanism in the brain and is associated with defective learning and plasticity (Donato et al., 2013). In the medial prefrontal cortex (mPFC) of Het, pyramidal neurons are hypo-responsive to interactions with a novel mouse or an object, and optogenetic inhibition of PV neurons rescues social deficits (Xu et al., 2022). Socially inhibited neurons in the prelimbic cortex are preferentially activated in response to a novel mouse, and this ability is impaired in MECP2-transgenic mice with overexpressed MECP2 (Zhao et al., 2022). Restoring MECP2 levels in prelimbic GABAergic interneurons rescues social novelty preference, suggesting appropriate expression of MECP2 regulates the ability to distinguish between old and novel social cues (Zhao et al., 2022). These studies collectively suggest mPFC functional hypoconnectivity in *Mecp2* mutant mice prevents discrimination between the context of social and nonsocial cues, ultimately leading to social behavioral deficits. Future work will focus on the role of mPFC in Het retrieval patterns.

While previous studies have reported regression in motor skills and respiratory function over weeks in *Mecp2* rodent models, it remains unclear when and how regression in social skills arise over time within Het (Achilly, Wang, et al., 2021; T.-W. Huang et al., 2016; Veeraragavan et al., 2016). Here, using multidimensional analysis of a social cognition pup retrieval task, we observed adult Het-R are capable of adapting goal directed trajectories towards pups in early days and regress as they are unable to consolidate or maintain these efficient trajectories in later days (**Figure 3.3**). Compared to WT, Het-NR have a milder phenotype but are capable of consolidating efficient trajectories over time. Thus, Het-NR are able to compensate behaviorally, while Het-R are unable to consolidate social and sensory cues to execute efficient retrieval. Het-R regression is specific to age as we do not observe regression in adolescent Het (Mykins et al., 2023). We observe adult Het have increased interactions with novel pup stimuli, and increased interactions with textures compared to WT controls, supporting Het may have issues in cortical circuits for discriminating social and nonsocial cues (Mykins et al., 2023; Stevenson et al., 2021). Severe cortical dysregulation in these circuits may result in the inability of Het-R to consolidate changes in sensory and social cues over time or prevent reinforcement learning of the reward of the pup (Xie et al., 2023), ultimately leading to regression. *In-vivo* optogenetic manipulation in the mPFC during maternal behavior are needed to discern whether Het are unable to distinguish between social cues of pups in later days, ultimately leading to regression. Furthermore, we have previously shown that in adult Het, pyramidal and PV neurons in the auditory cortex respond differentially to broad tones and pup calls, with lesser synaptic plasticity in the PV neurons (Lau, Krishnan, et al., 2020). The mPFC and auditory cortex reciprocally provide feedback for auditory tone discrimination, thus, optogenetic manipulation studies between these regions in the context of pup retrieval are needed to further dissect the sensory perception and motor activation pathology in Het (Rodgers & DeWeese, 2014).

Furthermore, cortical PV neurons are structurally surrounded by perineuronal nets (PNNs) which are specialized extracellular matrix structures that restrict synaptic

plasticity (Carstens et al., 2016, 2021; de Vivo et al., 2013; Donato et al., 2013; Durand et al., 2012; Gogolla et al., 2009; Hou et al., 2017; Kosaka & Heizmann, 1989; Krishnan et al., 2015, 2017; Lensjø et al., 2017; Morello et al., 2018; Nakagawa et al., 1986; Patrizi et al., 2020; Pizzorusso et al., 2002; Sigal et al., 2019; Thompson et al., 2018; Ueno et al., 2017). Abnormal PNN expression and PV expression are implicated in several mouse models for neuropsychiatric disorders (Carstens et al., 2021; Emery et al., 2022; Gandhi et al., 2023; Krishnan et al., 2015, 2017; Liu et al., 2023; Pizzo et al., 2016; Reinhard et al., 2019; Sultana et al., 2021). In a CNTNAP2 mouse model for autism, degradation of excess PNNs via chondroitinase ABC (ChABC) injection in the prefrontal cortex rescues social interaction deficits (Gandhi et al., 2023). Similarly, ChABC injection in the auditory cortex of Het partially rescues pup retrieval performance, indicating value in developing therapeutics targeted at PNNs (Krishnan et al., 2017). We also reported abnormal increase in PNNs in the primary somatosensory cortex of Het which coincides with atypical tactile interactions with textures, pups, and poor pup retrieval performance (Krishnan et al., 2017; Lau, Layo, et al., 2020; Mykins et al., 2023; Stevenson et al., 2021). New tools for *in-vivo* imaging of PNNs will aid in determining how changes in PNN expression may contribute to impaired integration of social and sensory cues, and ultimately regression (Benbenishty et al., 2023).

An alternative hypothesis is Het-R may lose the ability to transfer to the next task of bringing the pups back to the nest, suggesting issues in brain regions important for motor planning and execution. In support of this hypothesis, we reported that adult Het exhibit abnormal maternal behavior task transition probabilities during pup retrieval (Stevenson et al., 2021). The locus coeruleus is important for mediating maternal behavior in mice via global release of noradrenaline between transitioning of behavioral states, suggesting dysfunction in these circuits could contribute to inefficient pup retrieval in Het. *Mecp2* mutant mice have profound physiological deficits in the locus coeruleus (Dvorkin & Shea, 2022; Howell et al., 2017; T.-W. Huang et al., 2016; Taneja et al., 2009; Thomas & Palmiter, 1997). However, most of these studies focused on phenotypes in the context of respiratory dysfunction. Interestingly, DREAAD activation of mPFC pyramidal neurons ameliorate deficits in respiratory control in Het, suggesting

the reciprocal feedback between locus coeruleus and mPFC may be disrupted in Het and could contribute to sensorimotor and social communication issues (Howell et al., 2017).

Molecular and cellular mechanisms driving phenotypic variations in Het

Female patients typically survive into their middle age, exhibiting sensory processing, cognitive and motor deficits throughout life (Buchanan et al., 2019; Djukic et al., 2012; Djukic & Valicenti McDermott, 2012; Key et al., 2019; LeBlanc et al., 2015; Merbler et al., 2020; Neul et al., 2010; Nomura, 2005; Peters et al., 2015; Stallworth et al., 2019; Symons et al., 2019). In these females, random X-chromosome inactivation leads to mosaic wild-type MECP2 expression in approximately 50% of the cells, while the other 50% of cells will have deficient or no MECP2 protein. The random X-chromosome inactivation patterns across body and brain, and the type of MECP2 mutations are major contributing factors to the behavioral, cellular and molecular heterogeneity in Rett syndrome. Additionally, MECP2 protein in neurons is a stable, long-life protein, with half-life of around ~14days (Fornasiero et al., 2018; Mohar et al., 2022). Its expression increases in the postnatal brain and correlates with the timing of critical period plasticity of sensory cortices (Braunschweig et al., 2004; Durand et al., 2012; Krishnan et al., 2015; LaSalle, 2001; Picard & Fagiolini, 2019). Recently, we reported an intriguing observation of a precocious increase in MECP2 expression within specific cell types in the primary somatosensory barrel cortex of adolescent Het that coincides with typical behavioral performance during sensory relevant tasks (Mykins et al., 2023). We speculated this may provide compensatory phenotypic benefits in the earlier age, while the inability to further increase MECP2 levels in adulthood leads to regressive phenotypes (Mykins et al., 2023). Thus, mosaic MECP2 expression or the inability to maintain cell-type specific MECP2 expression may underlie behavioral regression in our Het-R phenotype. Further in-depth analysis of cell-type specific MECP2 expression within our adult Het-NR and Het-R phenotypes is needed. Previously, we also reported lateralization of MECP2 and PNN expression in the adult primary somatosensory cortex, which could contribute to functional specialization of the adult cortex, and is dysregulated in the Het (Lau, Layo, et al., 2020). Future work will focus on identifying

the nuanced expression and regulation of MECP2, an epigenetic master regulator with wide-ranging impact of gene expression.

Application of computational neuroethology tools to understand complex endophenotypes in neuropsychiatric disorders

Basic science research using behavioral endpoints in rodent models have identified key molecular therapeutic targets for treating core symptoms of Rett syndrome patients (Garré et al., 2020; Gogliotti et al., 2016, 2017; Gualniera et al., 2021; Li et al., 2017; O’Leary et al., 2018; Smith-Hicks et al., 2017; Tai et al., 2016). Recently, the FDA approved Trofinetide as the first drug for treating disease pathology in Rett patients (Neul et al., 2022). However, the challenge of predictive power to determine which therapeutic interventions will work when and how for individual patients is the holy grail of personalized medicine. In the preclinical stages, systematic characterization of etiologically relevant behaviors in female mouse models of X-linked disorders is critical to move forward in understanding the mechanisms of complex endophenotypes in patients and identifying the efficacy of clinical therapeutics.

The emergence of computational neuroethology tools now provides a unique opportunity to shift the paradigm from single constrained reductionist behaviors to studying complex behavior in free moving animals (Shemesh & Chen, 2023). The free availability of open-source marker-less deep learning and machine learning software for behavioral segmentation using pose makes it attainable and affordable to analyze complex free moving behaviors (Berman et al., 2014; Goodwin et al., 2022; Hsu & Yttri, 2021; Lauer et al., 2022; Luxem et al., 2022; Mathis et al., 2018; Nath et al., 2019; Nilsson et al., 2020; Pereira et al., 2019, 2022; Segalin et al., 2021; Sun et al., 2021; Wiltschko et al., 2015; Winters et al., 2022). Using unsupervised machine learning, multiple groups have identified subtle, novel behavioral differences in wild-type mice, and mouse models for Alzheimer disease, stroke, and Autism, highlighting the importance of these approaches for modeling complex endophenotypes of neurological

disorders in preclinical animal studies (Huang et al., 2021; Klibaite et al., 2022; Luxem et al., 2022; Segalin et al., 2021; Weber et al., 2022; Wiltschko et al., 2020).

As the field is broadening to include studies in female *Mecp2*^{heterozygous} mice, there is a need for efficient methods to assess phenotypic severity. Guy et al., (2007) established a severity scoring system, however, this system is specific to rapid phenotypic onset in null males and does not apply to female Het, which have a slow and progressive phenotypic onset and display considerable heterogeneity in behaviors. Additionally, this system requires extensive monitoring over time and introduces observer bias due to its qualitative nature. We highlight the advantage of using multidimensional analysis of behavior (**Figure 3.2**) for phenotypic characterization (**Figure 3.3**) and provide a means to derive a single value output (**Figure 3.2.g**) to score and evaluate Het phenotypic severity. The brevity and minimal resources for this approach make it an attractive approach to evaluate the efficacy of therapeutics in ongoing pre-clinical studies in the apt female Het mouse model. Future work will focus on clustering behavioral sequences or motifs, and manipulating neural circuitry during free moving behaviors, to determine which specific sensorimotor integration, and/or higher cognitive processes are affected during individual tasks and trials. Such studies will allow for precise testing and benchmarking of therapeutic and occupational interventions to treat neurological disorders throughout lifetime.

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Appendix

Table 3.1 Trajectory metric definitions.

Metrics:	Definition:
Latency index	- The amount of time to retrieve all pups back to the nest out of 5 min (retrieval phase of behavior only, excluded in habituation and isolation)
Errors	- Number of physical interactions with pup during the retrieval phase which do not result in retrieval, or dropping of the pup during retrieval (retrieval phase of behavior only, excluded in habituation and isolation)
Distance	- Total distance covered (in meters) during phase of behavior.
Duration	- The total amount of time it takes to retrieve all pups, or the max 5 minutes (retrieval phase) - For habituation, 5 minutes total - For isolation, 2 minutes total
Mean speed	- Change in distance over time (scalar quantity)
Mean acceleration	- Acceleration- change in speed over time (scalar quantity)
Mean step length	- The average distance between two steps
Sinuosity	- Tortuosity of a random search path (Benhamou, 2004; Bovet & Benhamou, 1988) - A function of both the mean cosine of turning angles and step length (Cheung et al., 2007; McLean & Skowron Volponi, 2018) - Varies between 0 (straight) and 1 (random motion)
Straightness	- Minimum distance to get from point a to b (D) divided by displacement from origin to final destination (L) (Batschelet, 1981) - Closer to 1, the more efficient the trajectory. The closer to zero, the more random the walk (Batschelet, 1981; Benhamou, 2004)
E _{max}	- Dimensionless value that denotes the maximum expected displacement of a random path as a function of the number of steps (Cheung et al., 2007; McLean & Skowron Volponi, 2018) - Values closer to 0 are more sinuous trajectories, larger values (approaching infinity) are straighter trajectories (Cheung et al., 2007)

Table 3.1 Continued.

Metrics:	Definition:
E _{max} b	- The product of E_{max} times the defined step size (Cheung et al., 2007) - larger values (approaching infinity) are straighter
Nonlinearity	- Measure of directional change - Angular change (in degrees) between two steps, divided by the time difference between the two steps (Benhamou, 2004; Kitamura & Imafuku, 2015)
Irregularity	- Standard deviation of directional change (nonlinearity) (Benhamou, 2004; Kitamura & Imafuku, 2015)

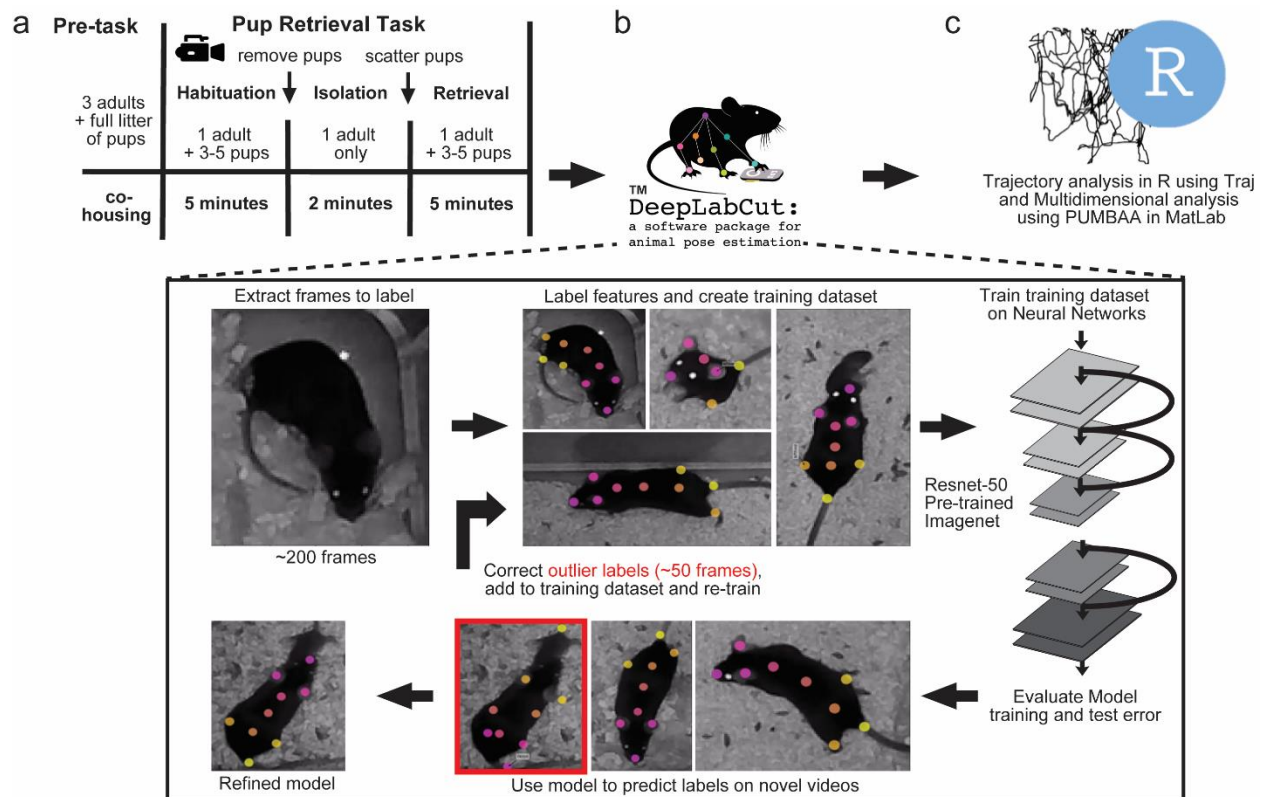


Figure 3.1. DLC derived pipeline for multidimensional analysis of trajectories.

(a) Schema depicting behavior setup. We performed and recorded habituation, isolation and pup retrieval in 6 weeks old (WO) adolescent and 10-12 WO adult female WT and Mecp2heterozygous (Het) mice for 6 consecutive days (see methods for details). **(b-c)** We trained videos of behavior in single animal DeepLabCut (DLC) **(b: top)** to predict pose of the animal. **(b: bottom)** features of interest were labeled and trained in DLC to build pose models which were then refined with more training to correct for errors. From our models we generate pose estimation and extract features of interest such as the nose to analyze trajectories of our mice in R using the Traj package (McLean and Skowron Volponi 2018) **(c)** and perform multidimensional behavioral analysis adapted from Tanas et al. 2022.

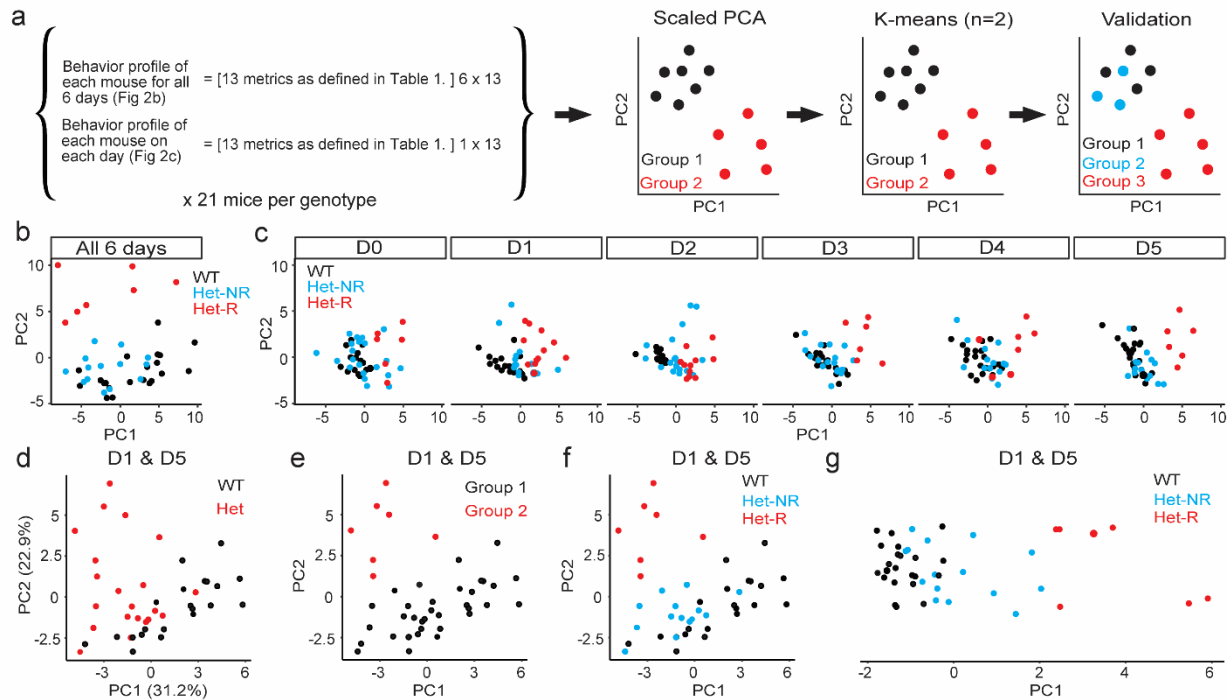


Figure 3.2. Multidimensional Analysis identifies two distinct phenotypes of Het.

(a) 13 trajectory metrics for all days of retrieval (78 total loadings) or 13 trajectory metrics on each day (13 total loadings) were standardized using z-scores for PCA (left). Example of analysis: each point represents one animal's behavioral profile in PC space, colored by Group (Scaled PCA). Mice were clustered into two groups (Group 1= black, Group 2=red) using k-means to predict genotype (K-means). The predicted genotypes were validated by comparing animals to their genotype (WT=black, Het predicted correctly = red, Het predicted incorrectly = blue) (Validation). (b) Multidimensional analysis for all days of retrieval revealed two groups of Het: Het that clustered with WT (Het-NR, blue) and Het that were distinctively different from WT and Het-NR (Het-R, red). (c) PCA, k-means clustering, and validation for each individual day of retrieval (D0-D5). (d) PCA of 13 trajectory metrics for D1 and D5 of retrieval (26 loadings) color coded by actual genotype (WT=black, Het=red). (e) K-means clustering (n=2) of 13 trajectory metrics for D1 and D5 of retrieval (Group 1= black, Group 2=red). (f) Validation of predictions supports two distinct groups of Het with reduced data complexity. (g) Principal component 1 is sufficient to separate the two distinct groups of Het. For all panels: each dot represents an animal, color-coded by genotype and phenotype. WT (black, n=21), Het-NR (blue, n=14), and Het-R (red, n=7)

Figure 3.3. Multidimensional analysis distinguishes Het that improve and regress in behavioral efficiency over days.

(a) Example trajectories of WT (black), Het-NR (blue) and Het-R (red) x-y nose position during pup retrieval towards the nest (purple box) across 6 consecutive days of behavior. **(b)** Variable correlation plots of trajectory metrics (see key below) from D1 and D5 represented in PC space. Variable vectors grouped in the same direction are positively correlated, and vectors in the opposite direction are negatively correlated. Variance explained (var. exp. %) represents the percent of variance explained by each PC component. **(c-d)** Percent contribution of top 15 (out of 26) metrics to Principal Component 1 **(c)** and Principal Component 2 **(d)**. The red dashed line represents the average expected contribution of each metric, everything above the red dashed line has considerable contribution to the component. **(e)** latency index, **(f)** Median distance nonlinearity and **(g)** Median distance traveled during pup retrieval for each test day showed significant improvement in retrieval trajectories across test days for Het-NR. However, Het-R improved over days and regressed by D5 compared to D0 and were worse than WT and Het-NR. WT (black, n=21), Het-NR (blue, n=14), and Het-R (red, n=7). Kruskal-Wallis followed by uncorrected dunn's test was performed to test for statistical significance between genotypes, and within genotype across days. Significance is plotted as correlation matrices colored coded by significance for **(e')** latency index, **(f')** nonlinearity and **(g')** distance. * p<0.05, ** p<0.01, *** p<0.001; **** p<0.0001, N.S. = not significant. **(b-d)** Trajectory metric key: number following abbreviation indicates the day of retrieval. [Dist: Distance, Dur: Duration, Err: Error, Ex: Emax, ExB: EmaxB, Irr: Irregularity, Lat: Latency, MAcc: Mean acceleration, MS: Mean speed, MSL: Mean step length, Non: Nonlinearity, Sin: Sinuosity, St: Straightness].

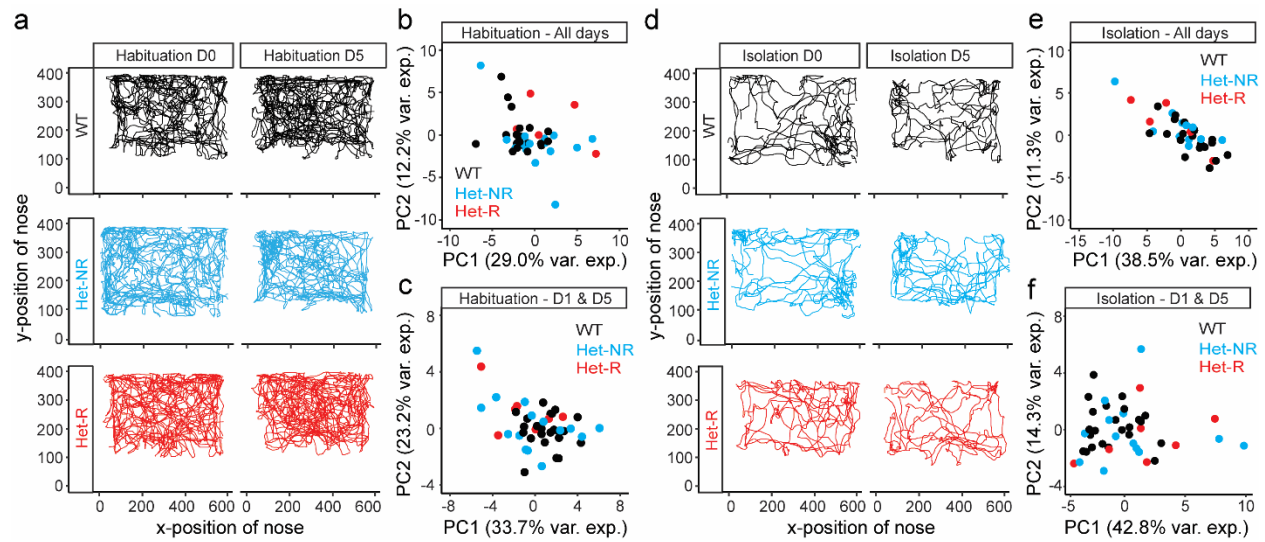


Figure 3.4. Regression is context dependent.

(a) Example trajectories of WT (black), Het-NR (blue) and Het-R (red) x-y nose position during habituation on D0 and D5. **(b-c)** PCA for all 6 days **(b)** and combined D1 and D5 **(c)** of habituation revealed no difference between identified groups during habituation (WT=black, Het-NR= light-blue, and Het-R=red). **(d)** Example trajectories of WT (black), Het-NR (blue) and Het-R (red) x-y nose position during isolation for D0 and D5. **(e-f)** PCA for all days **(e)** and combined D1 and D5 **(f)** of isolation revealed no difference between identified groups in the absence of pups. For **b-c, e-f**: each dot represents an animal, color-coded by genotype and phenotype. WT (black, n=21), Het-NR (blue, n=14), and Het-R (red, n=7).

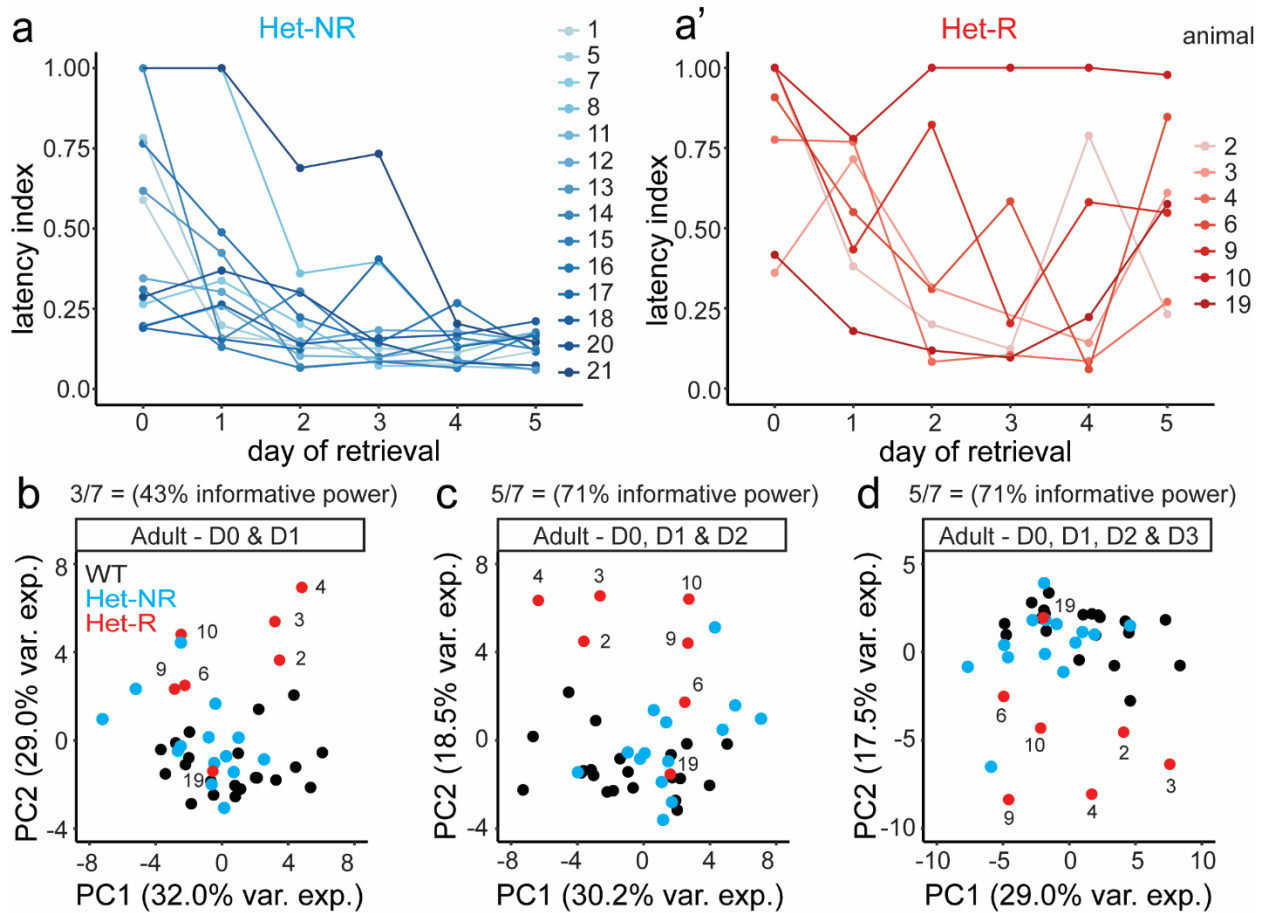


Figure 3.5. Performance in early days is able to identify future adult Het regressors.

(a-a') Individual animal trend lines of latency index for the identified **(a)** Het Non-Regressors (Het-NR) and **(a')** Het-Regressors (Het-R) from **Figure 3.2** and **2.3**. Trend lines are colored coded by animal number and highlight individual variation in latency index over 6 days for Het-NR (blue-gradient) and Het-R (red-gradient). **(b-d)** PCA analysis for D0 and D1 **(b)**, D0, D1 and D2 **(c)** and D0, D1, D2, and D3 **(d)** of retrieval. Analysis revealed that the first 3 **(c)** or 4 **(d)** days of pup retrieval had a strong accuracy of 71% for identifying 5 out of 7 Het regressors that regressed on D5 of retrieval, while the first 2 days **(b)** had a lower accuracy of 43% (3 out of 7 regressors). Informative power was determined by counting the number of D5 regressors within each cluster and dividing by 7 (the total expected number of regressors). Dots represent each animal [WT (black, n=21), Het-NR (blue, n=14), and Het-R (red, n=7)], and Het-R are numbered to easily identify regressors over time.

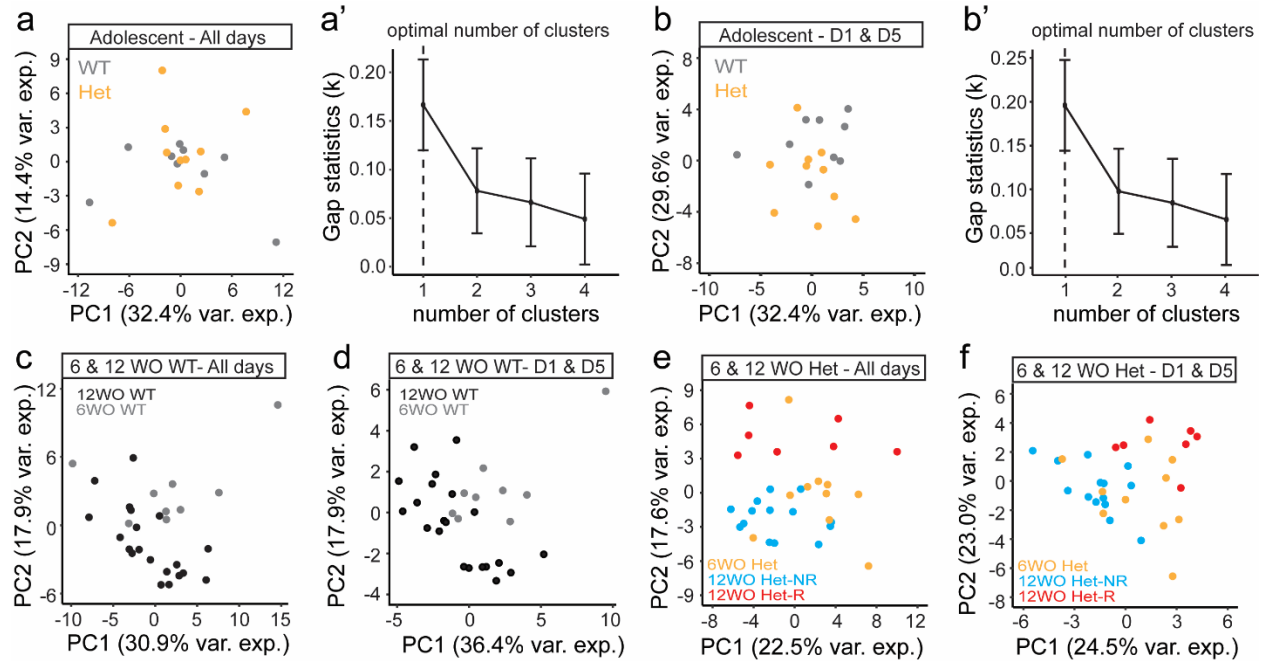


Figure 3.6. Het regression is age-specific.

(a) PCA for all days of retrieval, and (b) only D1 & D5 of retrieval reveals no difference in PC representation between 6-Week-Old (adolescent) WT and Het [WT (9) =grey, Het (11) =orange]. (a',b') Gap statistics test (see methods for details) confirms the optimal number of cluster is one (dashed line) for (a') all days of retrieval and (b') D1 & D5 of retrieval. (c) PCA for all days of retrieval, and (d) only D1 & D5 of retrieval reveals no difference in PC representation between adolescent WT (n=9, grey) and adult Het (n=21, black). (e) PCA for all days of retrieval of shows adolescent Het clusters with adult Het-NR. However, D1 & D5 of retrieval (f) shows adolescent He group with Het-NR and Het-R.

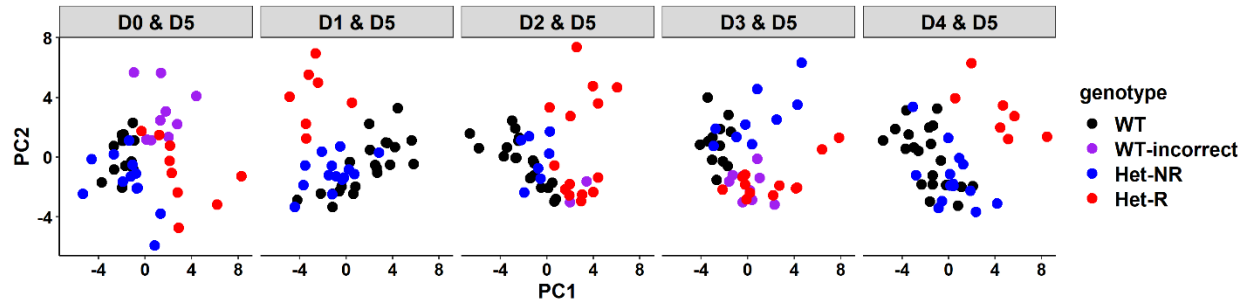


Figure 3.7. Multidimensional analysis of pairwise combinations with D5. Multidimensional analysis of pairwise combinations between (in order from left to right) D0:D5, D1:D5, D2:D5, D3:D5 and D4:D5 retrieval revealed combinations of D1 & D5 and D4 & D5 are sufficient to identify the Het-R group. Other combinations of days are insufficient because they incorrectly predict WT genotype (purple). (WT, black; WT-incorrect, purple; Het-NR, blue; Het-R, red). D0 & D5 (WT=12, WT-incorrect=9, Het-NR=12, Het-R=9), D1 & D5 (WT=21, Het-NR=14, Het-R=7), D2 & D5 (WT=19, WT-incorrect=2, Het-NR=8, Het-R=13), D3 & D5 (WT=14, WT-incorrect=7, Het-NR=10, Het-R=11), and D4 & D5 (WT=21, Het-NR=14, Het-R=7).

CHAPTER 4: ROLE OF PERINUEORNAL NETS IN THE SENSORY CORTEX

Abstract

MECP2 is a known regulator of synaptic plasticity in sensory critical periods during early postnatal development. Previously, we have shown that MECP2 also regulates inhibitory synaptic plasticity in adult auditory and somatosensory cortices, in an adult female mouse model for Rett syndrome (*Mecp2*^{heterozygous}, Het). MECP2 appears to regulate inhibition through parvalbumin+ GABAergic neurons (PV), which are surrounded by specialized extracellular matrix structures called perineuronal nets (PNNs). We found atypical increased PNN expression in primary somatosensory cortex (SS1), a brain region responsible for processing somatosensory information, of adult Het. We hypothesize that this atypical increased PNN expression leads to atypical tactile sensory perception in Het during contextually relevant behaviors. We tested this hypothesis by manipulating cortical PNN expression in wild-type and Het S1 and assessing their tactile and social behaviors. We use DeepLabCut to analyze pup retrieval videos for pose and trajectory of freely moving female mice, and DataVyu to analyze whisker interactions during texture discrimination assays. Systematic analysis of behavior revealed unilateral degradation of PNNs in the S1BF of wild-type did not affect consolidation of maternal behavior or texture investigation. Compared to control Het, bilateral or unilateral degradation of PNNs do not affect consolidation of maternal behavior or texture investigation. Overall, the findings suggest that appropriate expression of PNNs in the S1BF is not required for consolidation of maternal behavior or tactile processing during texture investigation.

Introduction

It is well known that there is lateralization in the human brain just as observed by the preference for being right-handed or left-handed. In lateralized circuits one hemisphere dominantly controls the processing and execution of sensorimotor commands. Lateralization in the brain is important for various cognitive and behavioral functions in humans such as speech and executive motor functions. Lateralized expression of diverse genes involved in synaptic transmission, signal transduction, glutamate receptor activity, nervous system development, and transmission of nerve impulses are observed postmortem human brain tissue (Kang et al., 2011; Karlebach and Francks, 2015; Pletikos et al., 2014). The specific contributions of these cluster of genes is unknown but suggest a dynamically regulated process is involved to control lateralization in the brain. Despite centuries of observations that laterality exist in humans, little is known about the neural mechanism that establish laterality in the brain. Recent studies have implicated lateralization of extracellular matrix structures called perineuronal nets, as a potential mechanism for establishing laterality in the rodent brain (Emery et al., 2022; Lau, Layo, et al., 2020).

Extracellular structures called perineuronal nets (PNNs) surround GABAergic neurons in the brain and are now emerging as substrates for learning and plasticity in the adult brain. Perineuronal nets (PNNs) are specialized extracellular matrix structures which structurally surround the soma and proximal dendrites of parvalbumin positive (PV+) GABAergic interneurons in the cortex (Hartig et al., 1994, 1992; Kosaka and Heizmann, 1989; Nakagawa et al., 1986). Most knowledge on the structure and function of PNNs is known from studies conducted in the visual cortex and hippocampus of rodents. PNNs restrict plasticity as their expression is concomitant with periods of heightened plasticity in the brain and enzymatic degradation of PNNs restores the brain to states of “juvenile” plasticity, highlighting a key role for PNNs in regulating synaptic plasticity (Hou et al., 2017; Krishnan et al., 2015; Patrizi et al., 2020; Pizzorusso et al., 2002b). However, little is known about the role of perineuronal nets in high cortical regions such as the somatosensory and motor cortex.

WFA and immunohistochemical staining of several PNN components show dense PNN structures in the somatosensory cortex of rodents (Billy Y. B. Lau et al., 2020; Mascio et al., 2022; Mykins et al., 2023; Thompson et al., 2018; Ueno et al., 2017, 2018). Enzymatic degradation of PNNs, via ChABC in the somatosensory cortex erases conditional learning of an auditory cue and tactile foot shock in trained adult WT mice (Thompson et al., 2017). Though it is unclear if this is because SS1 PNNs are important for the pairing of auditory and tactile stimuli, or if reducing PNNs disrupts perception of the tactile foot shock. Recently we reported that maternal experience induces hemispheric lateralization of PNNs in the somatosensory barrel field cortex (S1BF) which coincides with efficient pup retrieval in nulliparous adult female WT mice (Billy Y. B. Lau et al., 2020; Stevenson et al., 2021). Whether this lateralization is required for learned efficiency of pup retrieval is unclear.

Additionally, maternal experience does not induce lateralization of PNNs in nulliparous adult Het (Billy Y. B. Lau et al., 2020). Adult Het perform inefficiently at pup retrieval and have increased PNN density in the S1BF prior to behavior, suggesting the increased PNNs are inhibiting plasticity required to consolidate efficient pup retrieval. Interestingly, adolescent Het have similar PNN expression to wild type controls (**Figure 1.2**) and behave comparable to WT during pup retrieval (**Figure 1.3**) and tactile relevant tasks (**Figure 1.5**) (Mykins et al., 2023). Collectively, this suggests that abnormal PNN expression may impair tactile sensation, resulting in inefficiency during maternal behavior and tactile relevant tasks.

We hypothesized that appropriate expression of perineuronal nets in the somatosensory barrel field cortex is required for tactile processing in wild type. To test this hypothesis, we unilaterally injected Chondroitinase ABC (ChABC), an enzyme that degrades CSPG side chains of PNNs, into the right S1BF of adult WT and performed maternal behavior and texture discrimination task in our mice. Unilateral degradation of PNNs in adult WT (WT-uniChABC) did not affect pup retrieval performance or investigation of textures, suggesting that lateralized PNN expression is not relevant for these contexts in adult female WT. We next asked if atypical increase in S1BF PNNs in adult Het contributes to

tactile phenotypes in adult Het. We hypothesize that atypical PNN expression in S1BF impairs tactile processing leading to behavioral deficits in Het. We bilaterally injected ChABC into the S1BF of adult Het (Het-BiChABC) and ran mice through the maternal behavior paradigm and conducted the texture investigation task on the last day of pup retrieval behavior. Systematic behavioral analysis revealed bilateral injection of ChABC in adult Het (BiChABC) did not affect pup retrieval performance or texture investigation compared to control bilateral injection of penicillinase in Het (Het-BiPen). Compared to Het-BiPen, unilateral degradation of PNNs in adult Het (Het-uniChABC) did not affect pup retrieval performance or texture investigation, suggesting that abnormal S1BF PNN expression in adult Het does not contribute to our phenotypes.

Materials and Methods

Surgical injections

Mice were anesthetized with isoflurane (percent) and stabilized in a stereotaxic frame. To degrade PNNs, we injected chondroitinase ABC (0.1 ml of 50 U ml⁻¹ per site, in 0.1% BSA/0.9% NaCl solution; Sigma-Aldrich) into the somatosensory barrel field cortex of WT or *Mecp2*^{heterozygous} littermate mice. Penicillinase (0.1 ml 50 U ml⁻¹, in 0.1% BSA/0.9% NaCl solution; Sigma-Aldrich) was used as injection control. See appendix for detailed protocol of surgery setup. Mice were allowed to recover for two days and were cohoused with a pregnant mother 3-5 days prior to the mother giving birth. Specifics of injections are detailed in **Table 3.1**.

Behavior battery test

Pup Retrieval Task

The pup retrieval task was performed as previously described (Stevenson et al., 2021). Two 10-12-week-old naïve females (one WT and one Het) with no prior experience of pup retrieval were co-housed with a pregnant CBA/CaJ female (10-12 weeks of age) 3-5 days before birth; naïve females were termed ‘surrogates’ upon cohousing. Once the pups were born (postnatal day 0; P0), we performed the pup retrieval task with the surrogates for six consecutive days. The behavior was performed in the home cage placed inside of a sound- and light-proof box as follows: one surrogate was placed in

the home cage with 3-5 pups (habituation; 5 minutes), the pups were removed from the nest (isolation; 2 minutes), and the pups were scattered throughout the home cage by placing them in the corners and center, allowing the surrogate to retrieve pups back to the nest (retrieval; 5 minutes). The nest was left empty if there were fewer than five pups. The assay was performed in the dark and recorded using infrared cameras during all phases of each trial. If all pups were not retrieved to the nest by the 5-minute mark of the retrieval phase, we placed pups back in the nest in preparation for the next surrogate. After the behavior was completed, all mice were returned to the home cage. This was repeated for the first 4 consecutive days (D0-3), then on D4 and D5 we performed tactile discriminations tasks immediately after pup retrieval.

Behavior Analysis

All videos were coded so the analyzers were blind to the identity of the mice. Each video was manually scored using a latency index (the amount of time to retrieve all pups back to the nest out of 5 minutes) and the number of errors (surrogate-pup interactions that did not result in successful retrieval). Latency index was calculated as:

$$\text{Latency index} = [(t_1 - t_0) + (t_2 - t_0) + \dots + (t_n - t_0)] / (n \times L)$$

Where n = number of pups outside the nest, t₀ = start time of trial (s), t_n = time of the nth pup's successful retrieval to the nest (s), L = total trial length (300 s). Statistical analyses and figures were generated using GraphPad Prism.

Tactile Perception task

On day 4, immediately following maternal behavior, WT and Het underwent habituation to behavior chamber and testing on day 5 to test tactile processing. The behavior chamber used for tactile assays was a 40 cm x 40 cm x 40 cm plexiglass box with two sets of holes in the center of the plexiglass floor for the texture/object inserts. Holes were drilled to be equidistant from the center, with a 4.0 cm spacing between hole sets. Texture discrimination plexiglass inserts were 0.5 cm (thickness) x 4.0 cm (width) x 15.0 cm (height). Rodents use their whiskers to both detect and discriminate between textures and objects (Rodgers et al., 2021). Sandpaper textures were designed to slide

on and off the inserts to be cleaned between animals. The textures were made of 3M sandpaper of varying grit: a course 60-grit, and a fine 220-grit. The chamber and inserts were cleaned with MB-10 chlorine dioxide disinfectant and allowed to fully dry before the start of each behavior, and an additional 5-minute period was allotted between tests to eliminate scent biases. All behaviors were performed in the dark and recorded with an infrared camera.

Tactile Behavioral Paradigm

Habituation: On day 4 mice were placed individually into the behavior chamber for 10 minutes to habituate themselves with the new environment.

Texture Discrimination: On day 5, immediately following maternal behavior, texture discrimination assays began with a familiarization trial. Experimental mice were individually placed into the chamber with two identical texture inserts and allowed to investigate for 10 minutes. Afterwards, the mouse was returned to the home cage for 5 minutes. During this time, textures and inserts for familiarization were replaced with new sets of inserts and textures for use in the testing trial. After 5 minutes, the mouse was returned to the behavior chamber for a second 10-minute trial (testing phase). Familiarization phases of each behavior were performed using two course texture inserts. During testing phase on day 5 we replaced one insert with a fine texture.

Pose estimation generated from DeepLabCut:

For body part tracking we used DeepLabCut (version 2.2.0.3) (Mathis et al., 2018; Nath et al., 2019) We defined features of interest as the mouse's nose, left ear, right ear, shoulder, spine 1, spine 2, left hind limb, right hind limb, and tail base (**Figure 3.1.b**). We labeled 200 frames of features of interest taken from 25 videos then 95% of the frames was used for training. We used a ResNet-50-based neural network (Insafutdinov et al., 2016) with default parameters and trained for 720,000 iterations on Google Colab (Figure 1b). We performed outlier correction (**Figure 3.1.b**). to correct inaccurate predictions from the DeepLabCut model. In the GUI, we refined 50 frames of predicted labels by moving the predicted label's location to the actual position of the body part of

interest (**Figure 1b**). We then retrained, validated with 1 number of shuffles, and found the test error of was: 1.67 pixels, and train error was: 1.66 pixels (image size was 640 X 480 pixels). We then used a p-cutoff of 0.9 to condition the X, and Y coordinates for future analysis. This model network was then used to analyze novel videos of new adult animals from the same experimental settings.

Behavior Analysis

Behaviors for both texture and object investigation were manually scored using Datavyu software (Datavyu Team, version 1.3.7, 2014). All analyzers were blind to the age and genotype of the mice. The videos were scored for the time intervals in which the mouse interacted with the novel and familiar textures/objects throughout the 10-minute testing trial. We classified interactions into three categories, as shown in Table 1.1, based on pilot observations from previous studies (Mykins et al., 2023; Orefice et al., 2016; Pacchiarini et al., 2017; Wu et al., 2013). We excluded mice from analysis if the investigation time of textures/objects during a given trial did not exceed 2 seconds (Mykins et al., 2023; Orefice et al., 2016; Patrick Wu and Dyck, 2018; Wu et al., 2013) (**Table 1.2**). After manual scoring, interaction sequences for each animal were extracted from Datavyu for quantitative analyses. We classified number of contacts, total investigation time, and duration per interactions with textures/objects into whisker, forepaw, and whole-body climbing. Our results are reported as raw number of contacts, raw total investigation times, and raw duration per interaction split by interaction type, age, and genotype. We conducted unpaired Mann Whitney-U tests to determine statistical significance between genotypes and across ages within the same genotype. Statistical analysis and data visualization were performed in R studio.

Results

Experience dependent functional lateralization is observed in various sensory contexts such as auditory tone discrimination, observational fear learning, and fine motor learning in mice (Kim et al., 2012; Levy et al., 2019; Xu et al., 2009). In nulliparous adult female WT mice, novel exposure to pups induces experience dependent lateralized

changes in S1BF hemispheric PNN expression which coincides with efficiently pup retrieval (Billy Y. B. Lau et al., 2020). Whether this lateralization is required in the context of maternal behavior is not well understood. To investigate whether S1BF lateralization is required for efficient pup retrieval, we unilaterally degraded PNNs by injecting Chondroitinase ABC (ChABC) in the right hemisphere S1BF of adult WT and tested their performance in maternal behavior and texture discrimination (**Figure 4.1**). Compared to WT and WT-BiPen controls, unilateral degradation of S1BF PNNs in WT did not affect latency index or number of errors during retrieval on D0 and D5 (**Figure 4.2.a, b**). WT, WT-BiPen and WT-UniChABC latency index and number of errors significantly decreased between D0 and D5 (**Figure 4.2.a, b**). Overall, the findings suggest that lateralization of S1BF PNNs is not required for efficient pup retrieval in adult female WT mice.

We previously observed that consolidation of pup retrieval in WT is characterized by a decrease in the distance traveled, and a decrease in the directional changes during retrieval (**Figure 4.3.f, f', g, g' WT-black**). Thus, we used DeepLabCut derived pose estimation to quantify pup retrieval trajectories to determine if S1BF PNN expression is required for consolidating dynamics of pup retrieval. Compared to WT and WT-BiPen controls, there was no significant difference in the distance traveled or nonlinearity of pup retrieval trajectories on D0 and D5 (**Figure 4.2.c, d**). WT, WT-BiPen and WT-UniChABC distance traveled, and nonlinearity of pup retrieval trajectories significantly decreased between D0 and D5 (**Figure 4.2.c, d**). Collectively, the findings suggest that appropriate expression of S1BF PNNs is not required to consolidate efficient pup retrieval trajectories in adult female WT.

While tactile information is important for efficient pup retrieval, pup retrieval is a multisensory task which relies on other sensory systems such as audition and olfaction to localize and retrieve pups. Thus, other sensory systems may compensate in the absence of appropriate expression of S1BF PNNs. The main function of the S1BF is to process and decode incoming sensory stimuli from the whiskers. To test whether lateralization of S1BF PNNs is required for appropriate tactile processing, we performed

a texture discrimination assay on D4 and D5 of maternal behavior. Compared to WT-BiPen controls, unilateral degradation of S1BF PNNs in WT did not change the number of contacts, or time spent investigating textures with whiskers, forepaws or whole-body-climbing (**Figure 4.3.a-f**). Compared to WT-BiPen controls, unilateral degradation of S1BF increased the duration per interaction with forepaws (**Figure 4.3.h**), but not whiskers and whole-body-climbing (**Figure 4.3.g, i**). These results suggest that lateralization of PNNs does not affect overall texture investigation but increases the use of forepaws in adult female WT mice.

Surgery manipulation increases whiskers investigation in adult female WT

As we did not observe gross changes in texture investigation between WT-BiPen and WT-UniChABC, we thus asked how these mice compare to our previous report in un-manipulated adult female WT mice. Unexpectedly, compared to un-manipulated WT, surgery manipulation significantly increased the number of contacts, time spent investigating textures and duration per interaction with whiskers in both WT-BiPen and WT-UniChABC (**Figure 4.4.a, d, g**). Compared to un-manipulated WT, surgery manipulation did not affect use of forepaws in WT-BiPen (**Figure 4.4.b, e, h**), but significantly increased number of contacts, time spent investigating textures and duration per interaction with forepaws in WT-UniChABC (**Figure 4.4.b, e, h**). Compared to un-manipulated WT, surgery manipulation did not affect number of contacts and duration per interaction with whole-body climbing in both WT-BiPen and WT-UniChABC (**Figure 4.4.c, i**). Unilateral degradation of S1BF PNNs in WT significantly increased time spent investigating textures using whole-body climbing compared to un-manipulated WT (**Figure 4.4.f**). Overall, the findings suggest surgical manipulation, regardless of compound injected, increases the use of whiskers and is an important finding to consider when interpreting the results of the data. We speculate further on these findings in the discussion.

Het abnormal PNN expression in the S1BF is not causative of poor performance in sensory relevant tasks

In nulliparous adult female Het mice, there is increased S1BF PNN expression prior to behavior which coincides with inefficient pup retrieval, and tactile hyposensitivity during texture investigation (Billy Y. B. Lau et al., 2020; Mykins et al., 2023; Stevenson et al., 2021). We hypothesize that increased S1BF PNN expression inhibits processing of incoming tactile stimuli, resulting in efficient retrieval. To test this hypothesis, we bilaterally injected ChABC into the S1BF of Het and performed maternal behavior and a texture discrimination assay. After D5 behavior, we sacrificed and perfused the animals' brains for histology in order to confirm degradation of S1BF PNNs. Compared to Het-BiPen, ChABC treatment depleted expression WFA+ PNNs in the S1BF in Het-BiChABC (**Figure 4.5**). Compared to Het and Het-BiPen controls, bilateral degradation of S1BF PNNs in Het did not affect latency index during retrieval on D0 and D5 (**Figure 4.6.a**). Het, Het-BiPen and Het-BiChABC latency index significantly decreased between D0 and D5 (**Figure 4.6.a**). Regardless of experimental condition or day of behavior, there were no significant changes in number of errors during retrieval (**Figure 4.6.b**). Compared to Het and Het-BiPen controls, bilateral degradation of S1BF PNNs in Het did not affect distance traveled during retrieval on D0 and D5 (**Figure 4.6.c**). Het, Het-BiPen and Het-BiChABC traveled significantly less distance between D0 and D5 (**Figure 4.6.c**). Compared to Het and Het-BiPen controls, bilateral degradation of S1BF PNNs in Het did not affect nonlinearity of retrieval trajectories on D0 and D5 (**Figure 4.6.d**). Compared to Het and Het-BiPen controls, bilateral degradation of S1BF PNNs in Het significantly decreased nonlinearity of pup retrieval trajectories between D0 and D5 (**Figure 4.6.d**). Collectively, the findings suggest that abnormal S1BF PNN expression does not contribute to overall gross performance of pup retrieval but may contribute to subtle issues in sensorimotor navigation.

Previously we reported that adult Het have increased whisker, forepaw, and whole-body climbing interactions with textures which coincides with increased expression of S1BF PNNs (**Figure 1.9**) (Mykins et al., 2023). Interestingly, adolescent Het have similar S1BF PNN expression to WT control and investigate textures similarly to adolescent WT (**Figure 1.7**) (Mykins et al., 2023). Thus, we hypothesized that increased PNNs in

the S1BF inhibit the incoming whisker stimuli, and adult Het must interact more and use other peripheral modalities to compensate. Compared to Het-BiPen controls, bilateral degradation of S1BF PNNs in Het did not change the number of contacts (**Figure 4.7.a-c**), time spent investigating textures (**Figure 4.7.d-f**), or duration per interactions with whiskers, forepaws or whole-body-climbing (**Figure 4.7.g-i**). These results suggest that excess PNNs in the S1BF of Het do not contribute to the observed tactile hyposensitivity in adult Het.

Surgery manipulation exacerbates tactile hyposensitivity phenotype in Adult Het

As we did not observe any changes in texture investigation between Het-BiPen and Het-BiChABC, we thus asked how these mice compare to our previous report in un-manipulated adult Het. Compared to un-manipulated Het, surgery manipulation did not affect the number of whisker contacts in both Het-BiPen and Het-ChABC (**Figure 4.8.a**), but significantly increased time spent investigating textures and duration per interaction with whiskers in both Het-BiPen and Het-BiChABC (**Figure 4.8.d, g**), similar to findings in WT-Bipen and WT-UniChABC (**Figure 4.4.a, d, g**). Compared to un-manipulated WT, surgery manipulation significantly increased number of contacts, time spent investigating textures and duration per interaction with forepaws (**Figure 4.8.b, e, h**) and whole-body climbing (**Figure 4.8.b, e, h**) in both Het-BiPen and Het-BiChABC. Overall, the findings suggest surgical manipulation, regardless of compound, is exacerbating the previously reported adult Het hyposensitivity phenotype (**Figure 1.9**) (Mykins et al., 2023). Surgical manipulation itself is increasing whisker use in both adult WT and Het is not expected given the procedure. We speculate further on these findings in the discussion.

Inducing lateralized S1BF PNN expression exacerbates some tactile phenotypes in adult Het

Compared to adult female WT controls, adult Het lack lateralization of S1BF PNNs (Billy Y. B. Lau et al., 2020). We next asked if the lack of lateralization of PNNs in the S1BF of adult Het is contributing to the tactile hyposensitivity phenotype. To address this question, we induced lateralization in S1BF PNNs in adult Het by unilaterally degrading PNNs in the S1BF of the right hemisphere and injecting control penicillinase in the left

hemisphere. Compared to Het-BiPen controls, unilateral degradation of S1BF PNNs in Het did not change the number of contacts, or time spent investigating textures with whiskers, forepaws or whole-body-climbing (**Figure 4.9.a-f**). Compared to Het-BiPen controls, unilateral degradation of S1BF PNNs in Het significantly increased the duration of whisker and forepaw interactions (**Figure 4.9.g, h**) but not whole-body climbing interactions (**Figure 4.9.i**). Unexpectedly, unilateral degradation of S1BF PNNs exacerbated our previously reported phenotype. More studies are needed to confirm the findings.

Discussion

Functional lateralization in the cortex of rodents

Among other mammalian animals, rats and mice display functional lateralization in the brain. During observational fear learning in mice, unilateral inactivation and stimulation of the anterior cingulate cortex revealed only the right hemisphere is required for observational fear learning (Kim et al., 2012). Interestingly, inactivation of sub cortical thalamic projections to the cingulate cortex did not alter behavior suggesting that lateralization is localized to higher cortical regions. In motor cortex of adult male mice, learning of a fine motor skill induces increased dendritic spine density in the contralateral hemisphere of the dominant hand and is required for efficient pellet retrieval (Xu et al., 2009). In the somatosensory cortex of adult rats, there are individual hemispheric differences in the size of somatosensory barrel field cortex (Riddle and Purves, 1995). Whether there is a physiological function to these individual differences is not known. Previously, we reported that maternal experience induces lateralized expression of PNNs in the S1BF which coincides with efficient pup retrieval (Lau et al., 2020). Here we show that the lateralized shift in S1BF PNN expression is not required for efficient pup retrieval. Unilateral degradation of S1BF PNNs did not affect consolidation of efficient pup retrieval or texture investigation compared to control conditions (**Figure 4.2, Figure 4.3**). This suggest that appropriate S1BF PNN is not important in the context of maternal behavior or texture investigation. Enzymatic degradation of S1BF PNNs “erases” conditional fear learning of an auditory tone paired

foot shock, suggesting that the role of S1BF PNNs may be more context specific or important for tactile and auditory sensory integration (Thompson et al., 2018).

Disrupted lateralization in neurological disorders

Early work by Paul Broca in the late 1800s established that the left hemisphere of the brain is the dominant language center. In different neurological disorders this functional lateralization is disrupted. Functional imaging studies of the auditory cortex showed increased activation in the left hemisphere of patients without autism while there was increased activation in the right hemisphere of patients with autism (Eyler et al., 2012; Knaus et al., 2010). Changes in asymmetries in the anterior cingulate cortex and lateralized behavior are also observed in patients with cognitive and psychiatric disorders such as schizophrenia, attention-deficit/hyperactivity disorder, and bipolar disorder (Bush et al., 1999; Lindell and Hudry, 2013; Sommer et al., 2001). In addition, patients with Alzheimer disease show asymmetric buildup of amyloid beta in the cortex which positively correlated with severity of impairments (Frings et al., 2015). With the emergence of asymmetric and lateralized phenotypes there is thus a need for studying the mechanisms and physiological relevance of lateralization and its disruption in the brain. More invasive and manipulative approaches in animal models are needed to figure out the physiological relevance of lateralization in specific circuits within behavioral context.

Asymmetries and abnormal cortical PNN expression are observed in several mouse models for neuropsychiatric disorders such Autism Spectrum Disorders (ASD), Fragile-X syndrome, Schizophrenia, Alzheimer and Rett syndrome (Carstens et al., 2016, 2021; Emery et al., 2022; Gandhi et al., 2023; Krishnan et al., 2015, 2017; Billy Y. B. Lau et al., 2020; Liu et al., 2023; Pizzo et al., 2016; Reinhard et al., 2019; Sultana et al., 2021). In a CNTNAP2 mouse model for autism, degradation of excess PNNs via chondroitinase ABC (ChABC) injection in the prefrontal cortex rescues social interaction deficits (Gandhi et al., 2023). Similarly, ChABC injection in the auditory cortex of Het partially rescues pup retrieval performance, indicating value in developing therapeutics targeted at PNNs (Krishnan et al., 2017). We previously reported abnormal increase in

PNNs in the S1BF of Het which coincides with atypical tactile interactions with textures, pups, and poor pup retrieval performance (Krishnan et al., 2017; Lau et al., 2020; Mykins et al., 2023; Stevenson et al., 2021). Here, we report that bilateral degradation of excess PNNs in S1BF of adult Het does not rescue pup retrieval performance (**Figure 4.5**) and atypical tactile interaction with textures (**Figure 4.6**), suggesting increased S1BF PNNs are an indirect consequence of the underlying pathology causing sensory deficits in adult Het. An important factor to consider in interpreting these results is we only confirmed degradation of PNNs by using WFA (**Figure 4.5**). While ChABC results in loss of PNN structures as measured by WFA, several PNN components such as hyaluronan, hyaluronan proteoglycan link protein 1 (HAPLN1), tenascin-R, and brevican are present after ChABC treatment (Giamanco et al., 2010). To confirm excess or lack of lateralization of PNNs have no effect on impaired tactile behavior performance in adult Het, future studies should use multiple markers to confirm PNN degradation.

Caveats of surgical manipulation and alternative approaches to manipulate PNN expression

From the data it is clear that there is an effect on whisker texture investigation regardless of genotype (**Figure 4.4 & Figure 4.7**). Several studies have shown that isoflurane acts as a positive allosteric modulator of GABA-A receptors in neurons, though the mechanism of action is still unclear (Jenkins et al., 1999; Jia et al., 2008; Jones et al., 1992). It is unlikely that 90 minutes of exposure to isoflurane can have an effect approximately ~10-15 days later after the surgery. However, two hours of isoflurane (1.8%) exposure results in impaired conditional fear learning 12 days later in adult male C57/BL6 mice, suggesting there could be a post anesthetic effect of isoflurane (Yonezaki et al., 2015). What is more puzzling is that surgical manipulation significantly increased use of whiskers during texture investigation WT-BiPen controls (**Figure 4.4.a, d, g**), as previous studies do not see any effect of control penicillinase injections on WT mice. In other ChABC manipulation studies, control WT mice were anesthetized with intravenous tail injections of ketamine cocktail (Carstens et al., 2016;2021; Krishnan et al., 2017; Thompson et al., 2017), suggesting a potential effect of isoflurane on behavior in WT mice (Uchimoto et al., 2014). To test this hypothesis,

future experiments would expose adult WT and Het mice to isoflurane and test texture discrimination 12 days later or we would repeat experiments with a ketamine cocktail as the choice anesthetic. Alternatively, non-invasive approaches to manipulate PNN expression which do not require anesthetic, would circumvent these potential issues.

Several proof-of-concept approaches are available to degrade or remodel PNNs by targeting key components of PNN structure. A key component of PNNs is hyaluronan, which forms the backbone structure of PNNs, anchors PNNs to the neuronal membrane and allows binding of other important PNNs components such as link proteins (haplins), chondroitin sulfate proteoglycans (CSPGs) and tenascin. (Bruckner et al., 2000; Carulli et al., 2006; Fawcett et al., 2019; Fowke et al., 2017; Hartig et al., 1994; Kwok et al., 2010). Genetic Knockout of *Crtl1/Hapln1*, *Tenascin-R*, *Brevican* or *Aggrecan* significantly attenuates PNN structure (Brakebusch et al., 2002; Bruckner et al., 2000; Carulli et al., 2010; Romberg et al., 2013; Weber et al., 1999). More recent approaches now utilize chow infused with compounds that degrade or promote formation of PNN structure. 4-methylumbelliferone (4-MU) inhibits the synthesis of hyaluronan, and mouse chow infused with 4-MU reduces PNN density in WT mice (Dubisova et al., 2022; Kakizaki et al., 2004). Another study observed that microglia depletion via a drug infused chow increases PNN density in WT mice (Strackeljan et al., 2021). These alternative approaches provide non-invasive means to modulate PNN expression in order to determine context specific roles of somatosensory cortex PNNs.

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Appendix

Table 4.1 Detailed description for surgery manipulation conditions.

Genotype and experimental condition	Hemisphere (s)	Injected solvent	Coordinates of injection
WT-BiPen (n=13)	Bilateral- both left and right	Penicillinase- each site: 10 air puffs ~ = 0.15 uL 50U/mL compound	1) Bregma = -0.11 mm, lateral = 3.30 mm, depth = 0.5 mm 2) Bregma = -1.31 mm, lateral = 3.0 mm, depth = 0.5 mm
WT-UniChABC (n=14)	Left – penicillinase Right - ChABC	Left – penicillinase Right – ChABC each site: 10 air puffs ~ = 0.15 uL 50U/mL compound	1) Bregma = -0.11 mm, lateral = 3.30 mm, depth = 0.5 mm 2) Bregma = -1.31 mm, lateral = 3.0 mm, depth = 0.5 mm each site: 10 air puffs ~ = 0.15 uL 50U/mL compound
Het-BiPen (n=14)	Bilateral- both left and right	Penicillinase- each site: 10 air puffs ~ = 0.15 uL 50U/mL compound	1) Bregma = -0.11 mm, lateral = 3.25 mm, depth = 0.5 mm 2) Bregma = -1.31 mm, lateral = 2.75 mm, depth = 0.5 mm
Het-BiChABC (n=14)	Bilateral- both left and right	ChABC- each site: 10 air puffs ~ = 0.15 uL 50U/mL compound	1) Bregma = -0.11 mm, lateral = 3.25 mm, depth = 0.5 mm 2) Bregma = -1.31 mm, lateral = 2.75 mm, depth = 0.5 mm
Het-UniChABC (n=4)	Left – penicillinase Right - ChABC	Left – penicillinase Right - ChABC each site: 10 air puffs ~ = 0.15 uL 50U/mL compound	1) Bregma = -0.11 mm, lateral = 3.30 mm, depth = 0.5 mm 2) Bregma = -1.31 mm, lateral = 3.0 mm, depth = 0.5 mm

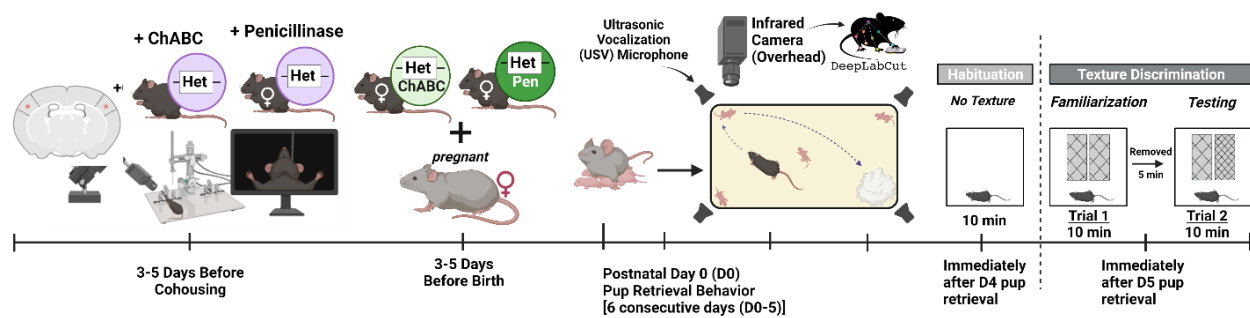


Figure 4.1. Behavior schema for ChABC manipulation and behavioral analysis.

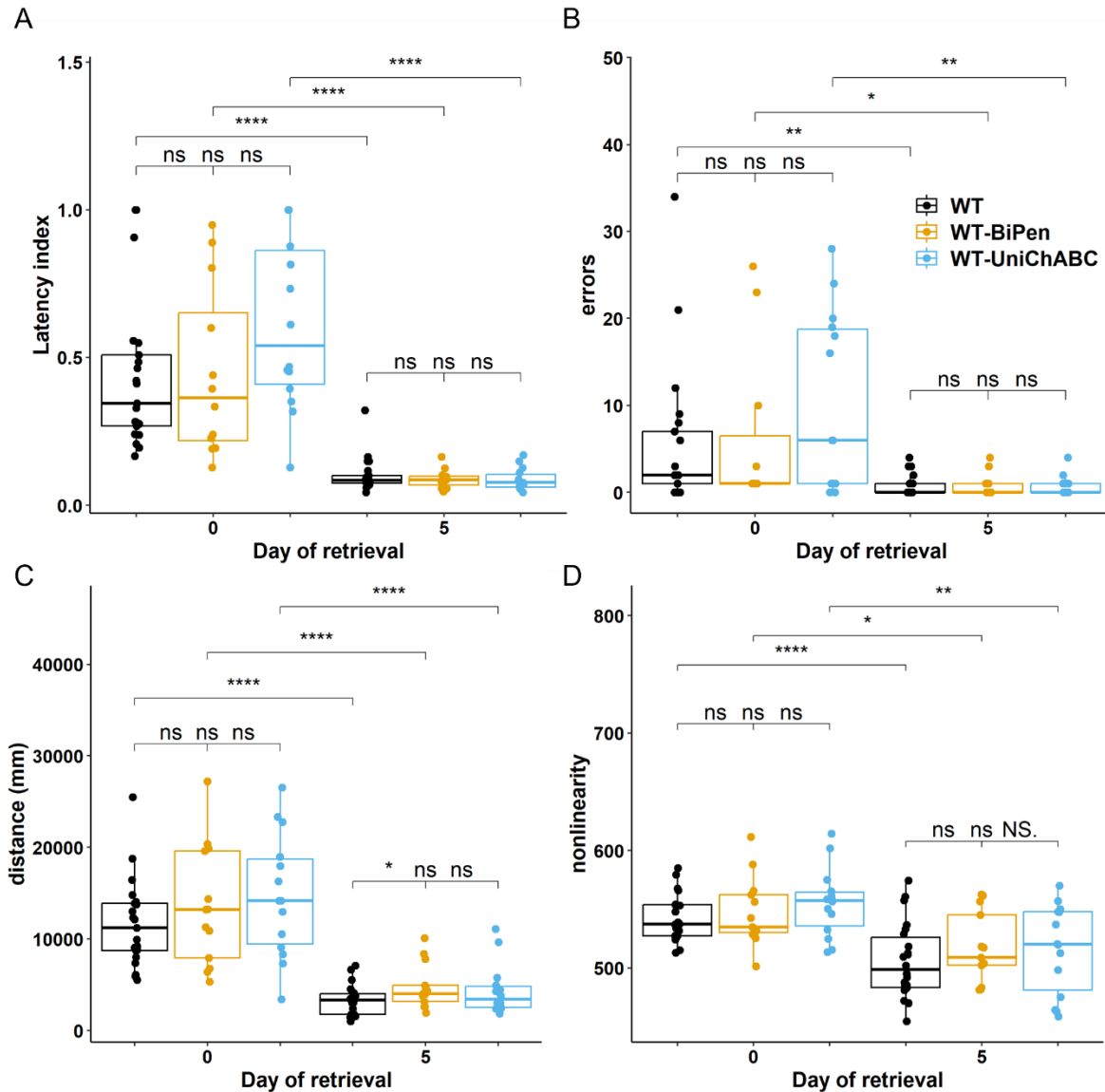


Figure 4.2 Lateralization of S1BF PNNs is not required for efficient pup retrieval in adult WT mice.

(a-d) Unilateral degradation of S1BF PNNs did not affect median latency index, number of errors, distance and nonlinearity of pup retrieval on D0 and D5 regardless of experimental conditions (WT: n=21 (black); WT-BiPen: n=13 (orange); WT-UniChABC: n=14 (light-blue)). Compared to performance on D0, WT, WT-BiPen and WT-UniChABC significantly improved in pup retrieval performance **(a-d)**. Boxplot represent the distribution of latency **(a)**, errors **(b)**, distance **(c)**, and nonlinearity **(d)**, and are color coded by genotype and manipulation. (WT: n=21 (black); WT-BiPen: n=13 (orange); WT-UniChABC: n=14 (light-blue)). Dots represent an individual animal on that day of retrieval. Kruskal-Wallis followed by uncorrected dunn's test was performed to test for statistical significance between genotypes, and within genotype on D0 and D5. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$, ns = not significant.

Figure 4.3 Unilateral degradation of S1BF PNNs does not affect texture investigation in WT mice.

(a-c) Statistical analysis of the interaction quantifications revealed adult WT-UniChABC (left S1BF hemisphere injected penicillinase; right S1BF hemisphere inject ChABC) interacted with textures using whisker, forepaw and whole-body climbing, similar to WT-BiPen (bilateral injection of penicillinase). WT-UniChABC had similar number of contacts **(a-c)**, total time **(d-f)** and duration per interactions with textures **(g-i)**. Superplots show animals color coded by circles, represent the mean of 2 trials (WT-BiPen, n=11; WT-UniChABC, n=11) and lines between samples represent animals whose surgeries were performed at the same time. Points on the plot are randomly shifted to the right or left to reduce overlap. Superimposition of violin plots show median and distribution of all interactions. Paired Kruskal-Wallis test determined no statistical differences.

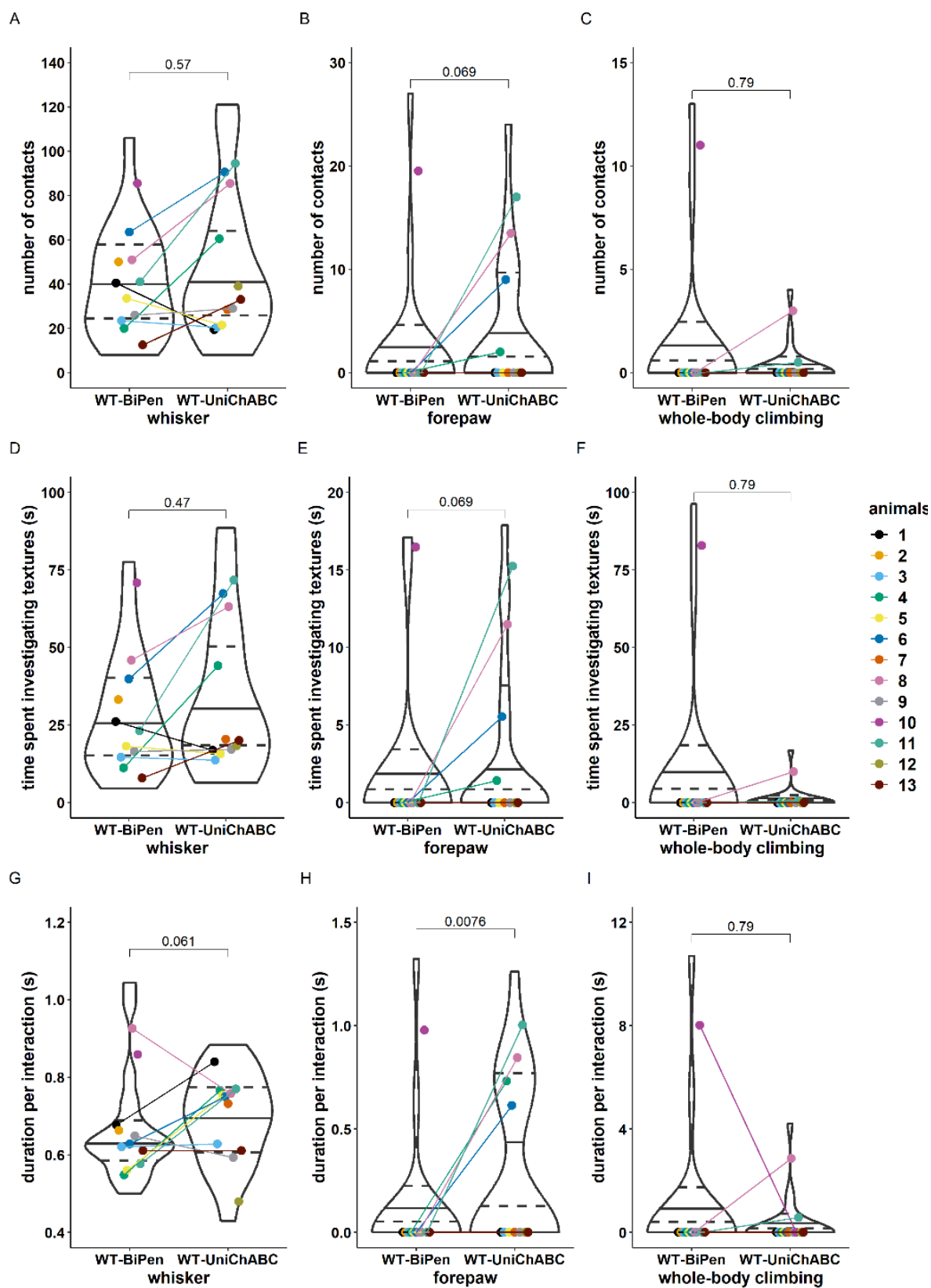
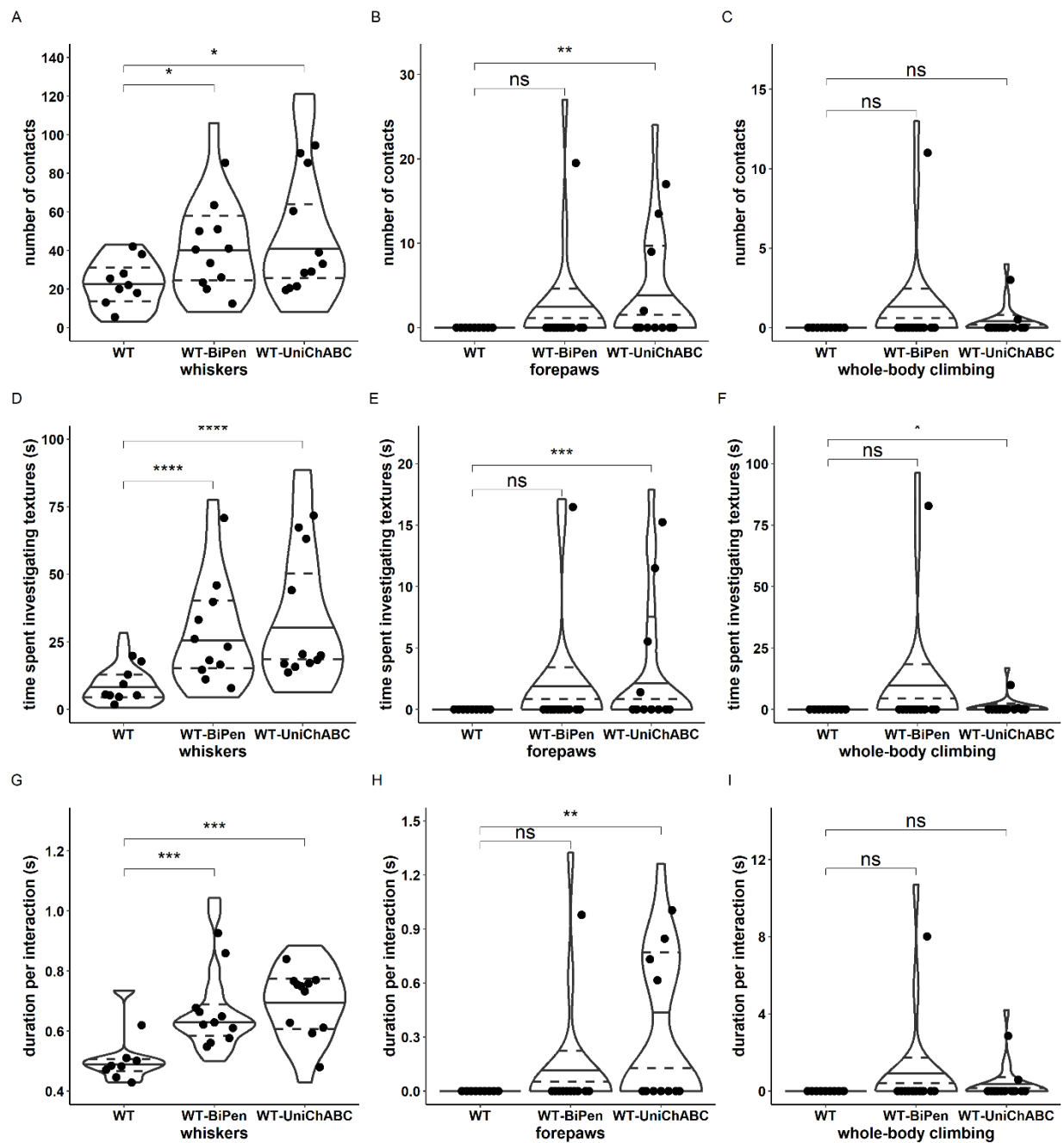


Figure 4.4. Surgery manipulation increase whiskers investigation in adult female WT

(a, d, g) Statistical analysis of texture interactions revealed adult WT-UniChABC (left S1BF hemisphere injected penicillinase; right S1BF hemisphere inject ChABC) and WT-BiPen interacted more with textures using whiskers compared to WT controls. WT-UniChABC had increased number of contacts **(b)** total time **(e)** and duration per interactions **(f)** using forepaws during texture investigation. Unilateral degradation of S1BF PNNs increased total time investigating textures during whole-body climbing **(h)** but not with whiskers and forepaw. **(h)** Superplots represent the mean of 2 trials (WT, n=9; WT-BiPen, n=13; WT-UniChABC, n=14). Points on the plot are randomly shifted to the right or left to reduce overlap. Superimposition of violin plots show median and distribution of all interactions. Unpaired Kruskal-Wallis test determined no statistical differences.



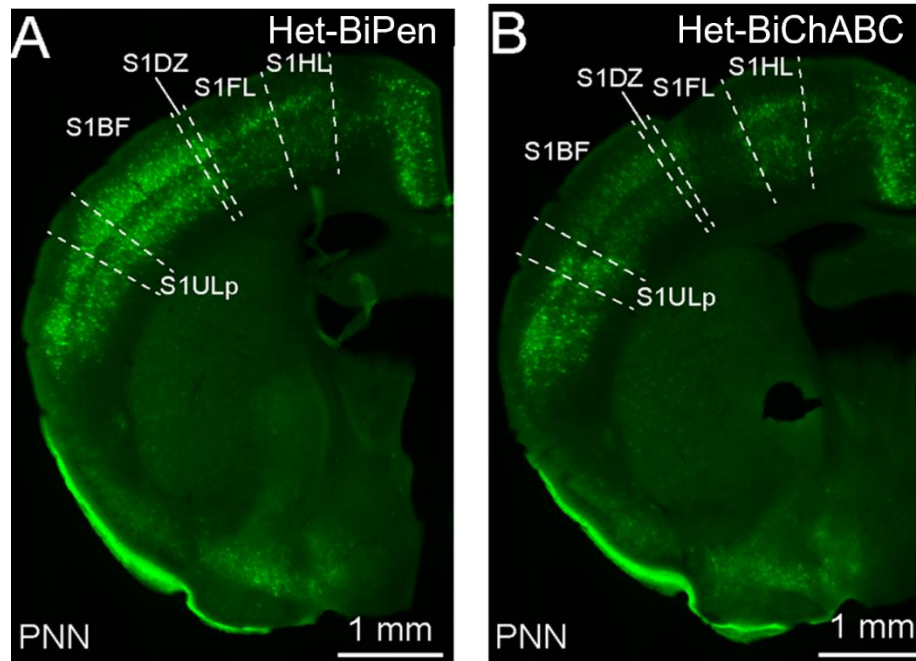


Figure 4.5. Immunohistochemical verification of degradation of PNNs in adult Het.

(a-b) Epifluorescent imaging of coronal brain slices from Het-BiPen **(a)** and Het-BiChABC **(b)**. Tissues were stained with Wisteria floribunda agglutinin to label perineuronal nets shown in green. Key brain regions of the somatosensory cortex are outlined (white dashed lines). Reduced WFA intensity in the S1BF of Het-BiChABC confirms depletion of chondroitin sulfate proteoglycan (CSPG) chains which form perineuronal structures.

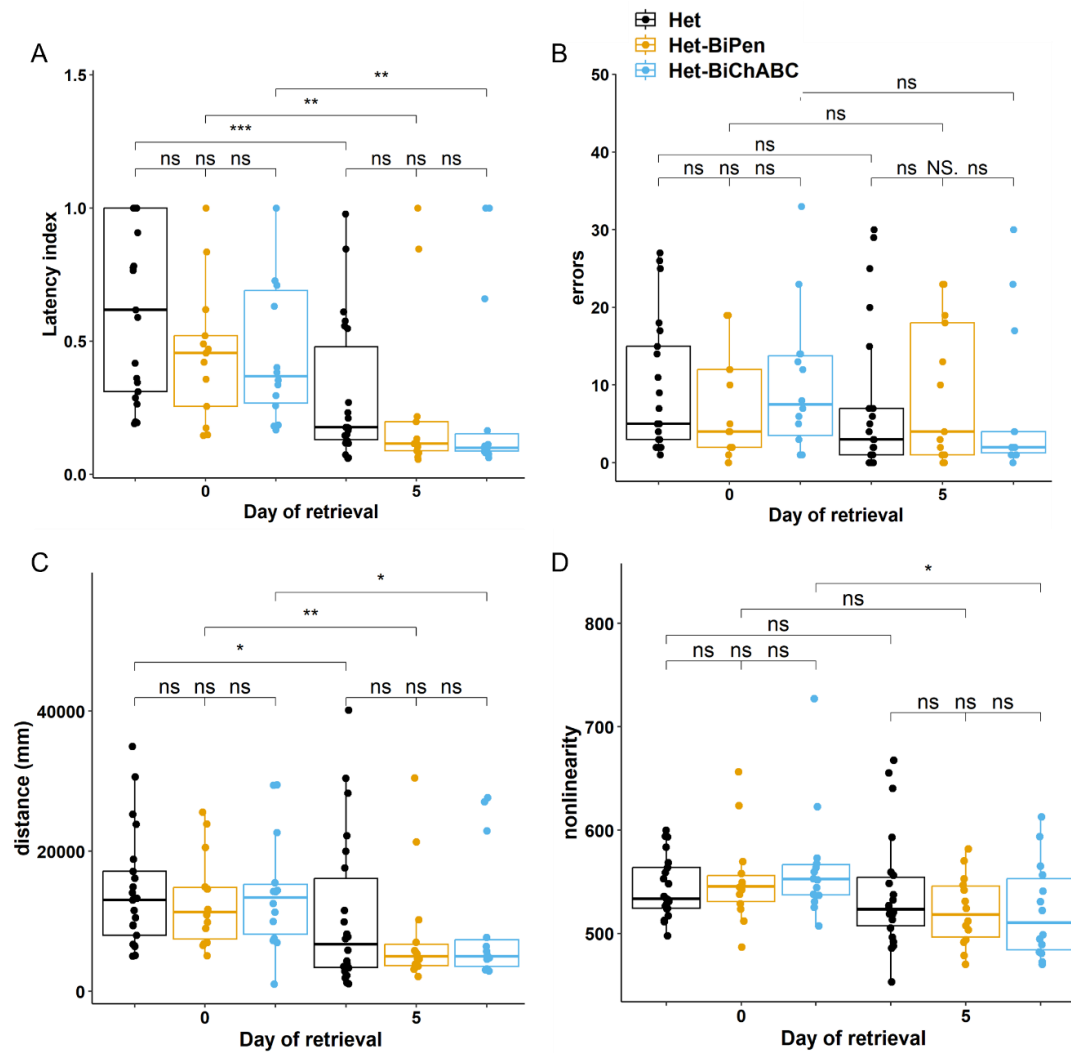
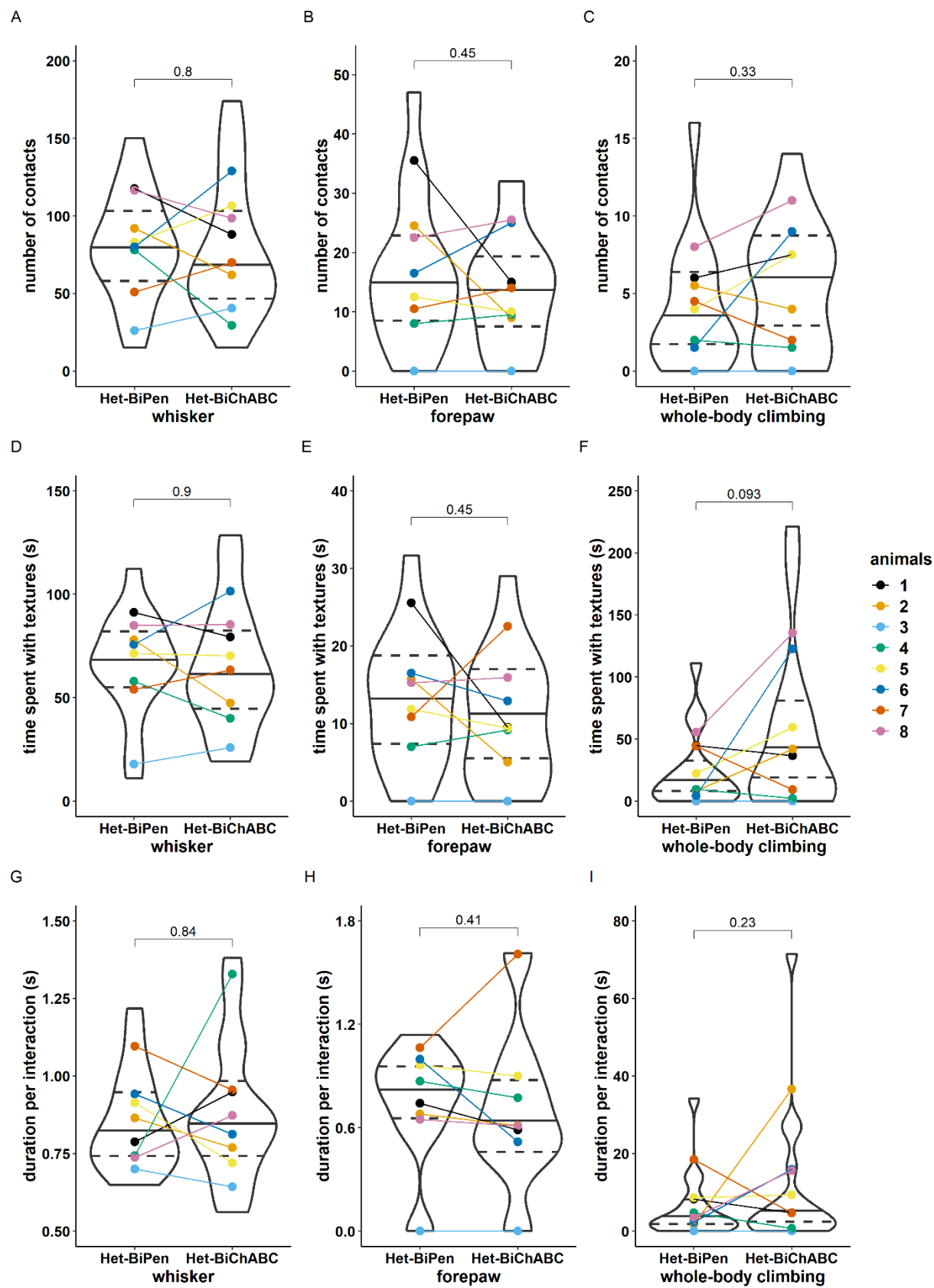


Figure 4.6. Excess S1BF PNNs do not inhibit consolidation of efficient pup retrieval in adult Het mice.

(a-d) Bilateral degradation of S1BF PNNs did not affect median latency index, number of errors, distance and nonlinearity of pup retrieval on D0 and D5 regardless of experimental conditions (Het: n=21 (black); Het-BiPen: n=13 (orange); Het-BiChABC: n=14 (light-blue)). Compared D0, Het, Het-BiPen and Het-BiChABC latency index and distance traveled significantly decreased **(a,c)** and number of errors did not change **(b)**. However, Het-BiChABC nonlinearity decreased significantly on D5 while Het and Het-BiPen showed no significant differences **(d)**. Boxplot represent the distribution of latency **(a)**, errors **(b)**, distance **(c)**, and nonlinearity **(d)**, and are color coded by genotype and manipulation. (WT: n=21 (black); WT-BiPen: n=13 (orange); WT-UniChABC: n=14 (light-blue)). Dots represent an individual animal on that day of retrieval. Kruskal-Wallis followed by uncorrected dunn's test was performed to test for statistical significance between genotypes, and within genotype on D0 and D5. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, ns = not significant.

Figure 4.7. Bilateral degradation of S1BF PNNs does not affect texture investigation in adult Het.

(a-c) Statistical analysis of the interaction quantifications revealed adult Het-BiChABC (Bilateral injection of ChABC in the S1BF) interacted with textures using whisker, forepaw and whole-body climbing, similar to Het-BiPen (bilateral injection of penicillinase in the S1BF). Compared to Het-BiPen, Het-BiChABC made similar number of contacts **(a-c)**, total time **(d-f)** and duration per interactions with textures **(g-i)**. Superplots show animals color coded by circles, represent the mean of 2 trials (Het-BiPen, n=8; Het-BiChABC, n=8) and lines between samples represent animals whose surgeries were performed at the same time. Superimposition of violin plots show median and distribution of all interactions. Paired Kruskal Wallis test determined no significant differences.



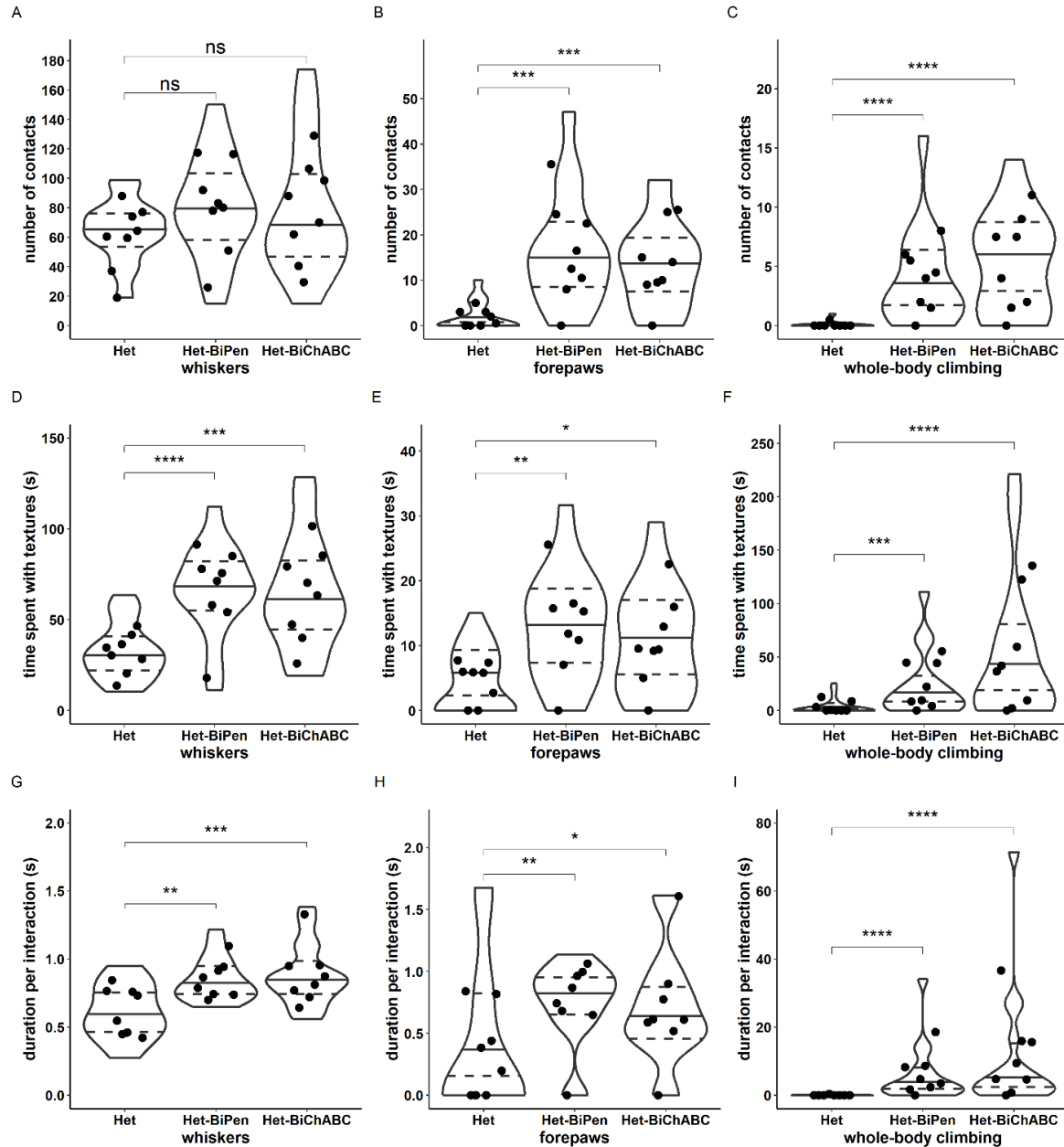
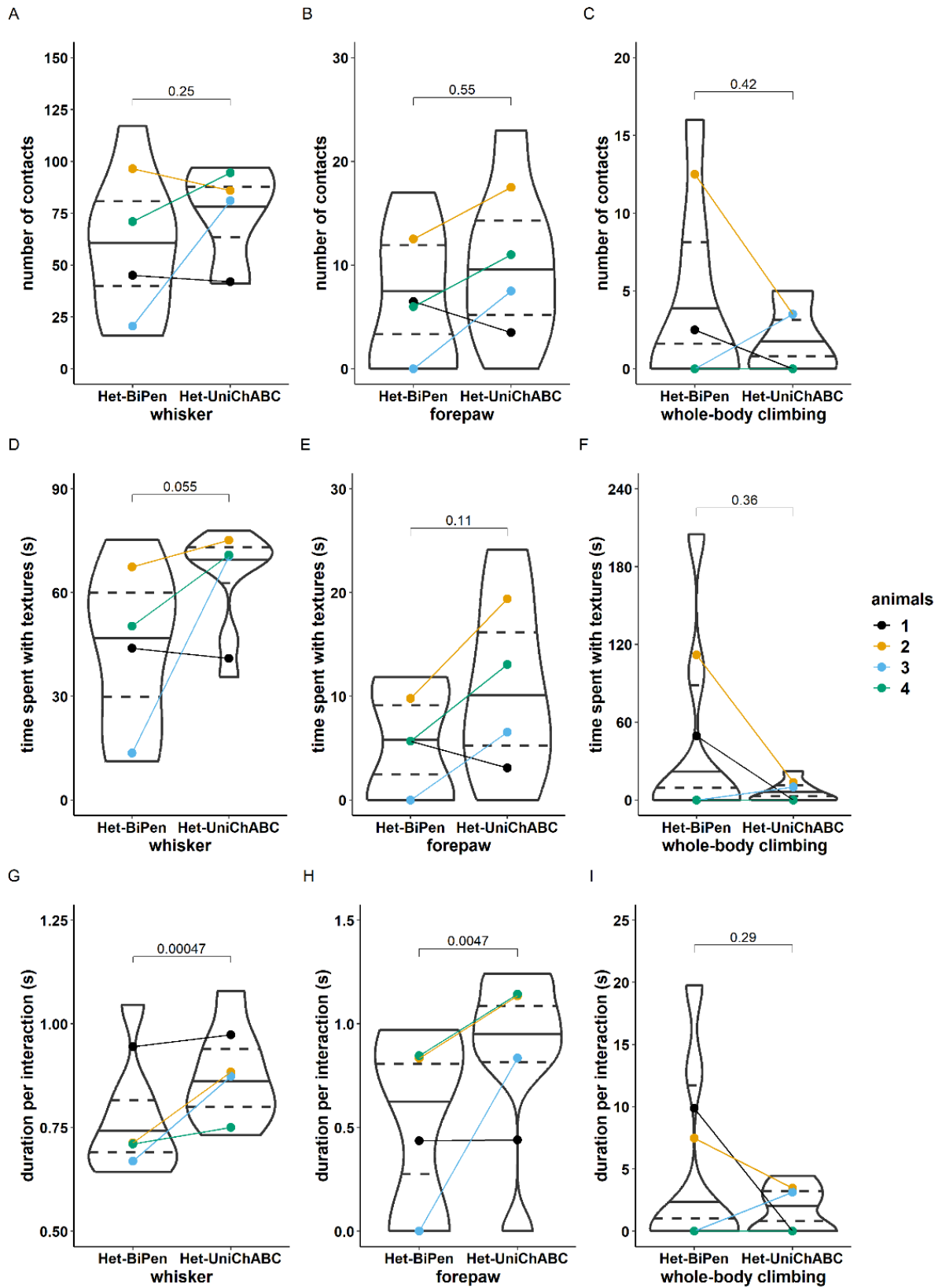


Figure 4.8. Surgery manipulation exacerbates tactile hyposensitivity phenotype in Adult Het

(a, d, g) Statistical analysis of texture interactions revealed surgical manipulation did not affect the number of whisker contact **(a)** but increased the time spent and duration per interaction with whiskers **(d, g)**. Surgical manipulation increased the number of contacts total time, and duration per interactions using forepaws and whole-body climbing during texture investigation **(b, c, e, f, h, i)**. Superplots represent the mean of 2 trials (Het, n=8; Het-BiPen, n=8; Het-BiChABC, n=8). Points on the plot are randomly shifted to the right or left to reduce overlap. Superimposition of violin plots show median and distribution of all interactions. Unpaired Kruskal-Wallis test determined no statistical differences.

Figure 4.9. Unilateral degradation of S1BF PNNs increases Adult Het duration of interaction with textures.

(a-c) Statistical analysis of the interaction quantifications revealed adult Het-UniChABC (left S1BF hemisphere injected penicillinase; right S1BF hemisphere inject ChABC) interacted with textures using whisker, forepaw and whole-body climbing, similar to Het-BiPen (bilateral injection of penicillinase in the S1BF). Het-Compared to Het-BiPen, Het-UniChABC made similar number of contacts **(a-c)**, and total time **(d-f)** during texture investigation. Compared to Het-BiPen, unilateral degradation of S1BF PNNs increased the duration per interactions with textures using whisker ($p=.00047$) and forepaw ($p=.0047$) **(g-h)** but not whole-body climbing **(i)**. Superplots show animals color coded by circles, represent the mean of 2 trials ($n=4$ Het-BiPen, $n=4$ Het-BiChABC) and lines between samples represent animals whose surgeries were performed at the same time. Superimposition of violin plots show median and distribution of all interactions. Paired Kruskal Wallis test was used to determine statistical significance.



CHAPTER 5: ESTABLISHING PARAMETERS TO INVESTIGATE FINE MOTOR LEARNING

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Abstract

Detailed analyses of overly trained animal models have been long employed to decipher foundational features of skilled motor tasks and their underlying neurobiology. However, initial trial-and-error features that ultimately give rise to skilled, stereotypic movements, and the underlying neurobiological basis of flexibility in learning, to stereotypic movement in adult animals are still unclear. Knowledge obtained from addressing these questions is crucial to improve quality of life in patients affected by movement related disorders such as Rett syndrome.

We sought to determine if known kinematic parameters of skilled movement in humans could predict learning of motor efficiency in mice during the single pellet reaching and grasping assay. We restricted mice of their food to increase motivation to reach for a high reward food pellet. Their attempts to retrieve the pellet were recorded for 10 minutes a day for continuous 4 days. Individual successful and failed reaches for each mouse were manually tracked using Tracker Motion Analysis Software to extract time series data and kinematic features. We found the number of peaks and time to maximum velocity were strong predictors of individual variation in failure and success, respectively. Overall, our approach validates the use of select kinematic features to describe fine motor skill acquisition in mice and establishes peaks and time to maximum velocity as informative measure of natural variation in motion efficiency in mice. We then validated the use of DeepLabCut to study fine motor learning in adult female WT and female mouse model for Rett syndrome.

Introduction

Early work in the 1980s established the use of the single pellet reaching assay for studying the acquisition of fine motor skill reaching in rodents (Evenden & Robbins, 1984; Whishaw et al., 1986). Rodents initiate reaching by lifting their forelimb from the ground, rotating their paw, advancing forward, clasping the pellet with their digits and returning the pellet back to their mouth (Nica et al., 2018; Whishaw & Pellis, 1990). Rodents are usually trained for 2-3 weeks, and their motor performance is reported as percent success after training. Traditionally, these studies used detailed qualitative assessments of the reach-to-grasp task by decomposing it into ten phases and manually scoring each phase independently (Gharbawie et al., 2005; Whishaw, 2000). However, these scoring systems require extensive time for analysis, training of individual rodents, and inter-observer validation to reduce observer bias and drift.

Most studies used overly trained rodents for circuit analysis and focus on end-point features (percent success, total attempts, and numbers of errors) (Schaar et al., 2010). Objective measurements that inform quality of motion are needed to improve our understanding of how a more efficient motion is learned with training. Natural variation in rodent motor performance is usually not reported, but there is considerable variability across individuals, ages, strains, and different litters (O'Bryant et al., 2011). Recent advances in deep learning and machine learning software are providing rich pose estimation for rodent reaching studies. With these advancements there is a push to focus more on individual variability vs stereotypies in behavior. We believe manual tracking of individual rodent reaching is needed to validate generated pose estimation and provide context in healthy and diseased models of motor disorders, such as Rett syndrome.

Symptomatic (14~16 Weeks-old) female mouse models of Rett syndrome have shared motor phenotypes with girls and women such as reduced mobility, and muscle rigidity. While it is clear that gross motor impairments are conserved between girls, women, and female mice, the neural circuitry deficits resulting in these motor deficits remains

unclear. Systematic characterizations of fine motor skills in pre-symptomatic (~12 Weeks-old) Het are needed to understand the how and when these motor deficits arise. We thus first sought to determine if known kinematic parameters of motion efficiency in humans could inform natural variation in fine motor efficiency in mice during fine motor learning of the single pellet reaching assay. We then validated the use of DeepLabCut for analysis of fine motor reaching strategies in female wild-type and Het mice.

Materials and Methods

Animals

Adult female wild-type C57BL/6 mice (8-10 weeks old) were either individually housed or group housed with littermates on a 12hr light-dark cycle (lights on at 07:00 AM) and received food ad libitum until start of the procedure.

Behavioral paradigm

At the start of the procedure, mice were placed on a calorie-restricted diet, in order to achieve a 10 percent reduction in their body weight. Water access was maintained 24/7 throughout this period. During dietary restriction, the weights of the mice were monitored each day. Once mice reached ~90 percent of their original body weight (after 9-12 days), they were introduced to the single pellet reaching assay chamber. Each day mice were placed in a Plexiglas chamber (lwh (cm): 20.3 X 8.3 X 21) containing a narrow-slit opening (width: 5 mm) towards an automatic pellet dispensing platform operated by an actuator (Fig. 1). An automatic pellet dispenser releases a pellet which is then brought up on a platform containing an infrared sensor. Any motion detected by movement to retrieve the pellet by the mouse will result in closure of the slit by a sensor activated door and lowering of the platform containing the pellet. After 10 seconds, the platform is raised and the door lifts to allow continued attempts to retrieve the pellet. Mice were placed in the apparatus and given 10 minutes to retrieve as many pellets as they can. During this time, mice were recorded using a Blackfly USB 3.0 camera (FLIR Systems Integrated; Wilsonville, Oregon). Videos were collected in SpinView (FLIR Integrated Systems) at 200 frames per second (FPS), with a 1000 μ s exposure time and 628x647 pixel resolution. Observers recorded and scored each attempt to retrieve a

pellet as a success (the mouse successfully grasps the pellet and brings it to the mouth) or failure (the mouse attempts to retrieve the pellet but does not bring it back to the mouth). Behaviors were performed between 9 AM-6 PM and this process was repeated for a total of 4 days of exposure for 5 animals. All procedures were in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and approved by the University of Tennessee-Knoxville Institutional Animal Care and Use Committee.

Manual Kinematic analyses of goal-directed reaching

Video data acquired during pellet reaching were imported into the free, open-source Tracker motion analysis software (<https://physlets.org/tracker/>, Copyright © 2020 Douglas Brown, Robert Hanson, and Wolfgang Christian). All attempts, excluding errors (when a mouse reached in the absence of a pellet) were manually tracked and recorded from motion onset to offset. Motion onset was defined as the initiation of reach from a resting home position (i.e., the paw immediately before lifting from the ground). Motion offset was defined as the timepoint at which the pellet was brought to the mouth (successful attempt) or the return of the paw to the ground (fail attempt). Before analysis, a length scale was set in the Tracker by measuring the distance between the chamber opening and platform (~ 6 mm). Of the total 428 tracked reaches, 57 multiple reaches (3 or more attempts without returning the paw through the gate) were removed from further analysis as outliers. Video coders made note of the limb preference. The majority of mice used both limbs initially and emerged as left limb dominant. Paw location data (x and y position coordinates) extracted from Tracker were exported to MATLAB (R2019a) for further analyses. These extracted text files were named according to animal, type, trial number, and outcome (e.g., success or failure). Raw position data were differentiated to obtain velocity, acceleration, and jerk in the x and y directions. In addition, the resultant position, velocity, and acceleration were calculated using root mean square. A variety of kinematic features were then extracted from these time series data. The selected outcome measures included: the number of peaks, maximum velocity, time to maximum velocity, and mean squared jerk.

Measures were computed using standard definitions. The number of peaks were determined by summing instances when the derivative of the position data was equal to zero. Maximum velocity was determined by finding the maximum value from the resultant velocity time series. Time to maximum velocity was determined by subtracting the time of movement onset from the time corresponding to maximum limb velocity. Mean squared jerk was calculated using time normalized jerk:

$$\text{Mean squared jerk} = \frac{1}{\Delta t} \int_{t_0}^t \sqrt{x'''(t)^2 + y'''(t)^2} dt$$

Where x''' and y''' represent the third derivative with respect to time for the x and y paw coordinates (respectively), and Δt represents the time between sample frames. Analyses and data visualization were performed in R studio (R version 4.0.3). An unpaired Kruskal-Wallis with Dunn's method was performed to test for statistical significance between number of peaks, time to maximum velocity, maximum velocity, and mean squared jerk of successful and fail reaches over days of exposure. Correlation plots, liner regression analyses, and sequence plots were generated in R studio to analyze the relationship between efficiency and days of exposure.

Automatic kinematic analysis of goal-directed reaches

Video acquisition:

Videos were acquired from fine motor training experiments at Cold Spring Harbor Laboratory. 10-minute videos were trimmed to extract the max number of successes, and equal or less number of failed attempts based on percentage of success. For example, if percent success was 40%, with 50 attempts, then 20 successes and 20 failed attempts were trimmed for that animal. For day 3, 8, and 14 for WT (n=4) and Het (n=6), this resulted in a total of 1012 reaches extracted. Details per individual animal are found in Table 4.3. These videos were then used for training and analysis in DeepLabCut.

Pose estimation generated from DeepLabCut:

For body part tracking, we used DeepLabCut (version 2.2.0.3) (**Figure 5.6**). We defined features of interest as the nose, rightbodypart1 (right wrist), rightbodypart2,

rightbodypart3, rightbodypart4, leftbodypart1, leftbodypart2, leftbodypart3 and leftbodypart4 (**Figure 5.6**). We labeled 200 frames of features of interest taken from 10 videos of WT and Het during fine motor training and then 95% of frames were used for training. We used a ResNet-50-based neural network with default parameters and trained for 550,000 iterations on Google Colab (Insafutdinov et al., 2016). We performed outlier correction to correct inaccurate predictions from the DeepLabCut model. In the Graphical User Interface (GUI), we refined 60 frames of predicted labels by moving the predicted label's location to the actual position of the body part of interest (**Figure 5.6**). We repeated this process until we achieved a test error of 1.27 pixels, and train error of 1.1 pixels (image size was 640 X 480 pixels). We then used a p-cutoff of 0.9 to condition the X and Y coordinates for future analysis. This model network was then used to analyze novel reaching videos.

Pose estimation derived reaching trajectory analysis:

To quantify reaching trajectories across training within each genotype, we used the Traj R package (McLean & Skowron Volponi, 2018). We filtered the X and Y coordinates of the right wrist from the DLC pose and quantified the mean speed and straightness of reaching attempts for each genotype across training. Mean speed and straightness were extracted as previously described in Chapter 2 (**Table 2.1**).

Results

To determine if kinematic parameters used in human studies can predict motor learning efficiency in mice, we analyzed parameters during the initial exposure to the single pellet reaching task in adult female mice. These parameters include the number of peaks, maximum velocity, time to maximum velocity, and mean squared jerk. During the single pellet-reaching assay, mice were food restricted to increase motivation to reach to a high reward food pellet. Mice were recorded during behavioral performance, and pellet reaching was manually recorded using Tracker, an open-source tracking software. We then derived relevant kinematic parameters to determine the relationship between these established parameters and motor efficiency in mice. We hypothesized

that if patterns of motor output during a reach to grasp task were homologous between humans and rodents (Karl & Whishaw, 2013; Klein et al., 2012; Sacrey et al., 2009), then known human kinematic parameters can predict motor efficiency in mice.

Peaks are an informative measure for individual variation in percent failure measurement

The numbers of attempts, successes, and fails were manually scored to generate the average percent success efficiency across four days (**Figure 5.2.a**). On average, the percent success in adult female mice increased over time, which is consistent with previous observations in rats and mice (Buitrago et al., 2004; Chen et al., 2014). There was natural variability in performance during the early skill acquisition phase of single pellet retrieval. Some animals started out as fast learners or non-learners as previously defined (Chen et al., 2014). Animals 1 & 2 performed better initially and leveled off compared to animals 3 & 4, which started off poorly and got better with exposure. Animal 5 performed poorly and did not achieve a high percent success compared to the other animals.

In human studies, peaks (a measurement of movement intermittency) are predictive measures of motor efficiency (Balasubramanian et al., 2015; Berthier & Keen, 2006; Mancini et al., 2011; Rand et al., 2000; Teulings et al., 1997). Peaks in position, also referred to as sub-movements, are defined as in acceleration ‘zero-crossings’, between accelerating and decelerating phases of motion (Rohrer et al., 2002, 2004). The number of peaks is inversely related to efficiency and smoothness. In our study, the average number of peaks in successful reaching did not change with additional exposure (**Figure 5.2.b**). However, the average number of peaks in failed reaches decreased significantly between Day 1 (D1) and Day 4 (D4). By D4, there was not a difference in the average number of peaks between successful versus failed reaches, indicating a more efficient motion (**Figure 5.2.b**). Linear regression analyses showed a significant positive correlation between percent failure and the average number of peaks for failed reaches ($r^2=0.348$, $p\text{-value} < 0.01$) (**Figure 5.2.c**). There was no correlation between percent success and the average number of peaks ($r^2=0.224$, $p\text{-value} > 0.05$). Individual animal correlation of average peaks in failed reaches to percent failure was a

strong predictor of natural variation in percent failure (**Figure 5.2.d**). To capture natural variation, we examined the distribution of peaks for failed reaches and successful reaches. The distribution of peaks for successful reaching did not change across days (**Figure 5.2.e, bottom panel**). The distribution of peaks in failed reaches decreased across days, most notably for animals 3 & 4 (**Figure 5.2.e, top panel**), also seen concomitantly with percent failure (**Figure 5.2.d**). For animals 1 & 2, we did not observe similar patterns, likely due to their relative efficiencies in successful reaching of the pellets, and relatively low number of peaks compared to other animals. The distribution of peaks increased with days for animal 5, consistent with inefficient pellet reaching. Thus, the more efficient a mouse is at pellet reaching, the more likely it is to have a low number of peaks in its reaching strategy. This result is consistent with observations in human studies that demonstrated peaks decrease with motor learning and improved motor function (Berthier & Keen, 2006)

Mice correct the number of peaks in their reaching strategies after failure

In humans, failed attempts in a reaching task are followed by corrective strategies to increase success (Taylor et al., 2014). With continued attempts in trials, subsequent movements in a reaching task are less variable suggesting a corrective feedback mechanism for cognitively improving performance in reaching in humans and mice (Becker et al., 2020; Duthoo et al., 2014; Verstynen & Sabes, 2011). We plotted the sequences of number of peaks for all attempts during the 10-minute trial across days (**Figure 5.3**). On D1, there is considerable variability in the sequence of number of peaks for all animals. On subsequent days, for animals 1-4, failed reaches with high number of peaks (warmer colors) are followed by attempts with a lower number of peaks (cooler colors). As we might expect, this observation was not observed in animal 5 which performs poorly at the task. Thus, mice may use sensory feedback from previous failed attempts to adapt a more efficient reaching strategy in subsequent trials.

Time to maximum velocity is an informative measure for individual variation in percent success

In humans, time to maximum velocity and maximum velocity, also known as time to peak velocity and peak velocity, decrease as a motion becomes more efficient (Ma &

Trombly, 2004; Wang et al., 2018; Wu et al., 2000). Maximum velocity of reaching did not change between success vs fail reaches in our mice (**Table 5.1.**). Time to maximum velocity (s) decreases significantly between D1-D4. Time to maximum velocity of successful reaches and fail reaches decrease significantly across days (**Figure 5.4.a**). Linear regression analyses showed a significant negative correlation ($r^2=0.3717$, p -value < 0.01) between percent success and average time to maximum velocity (**Figure 5.4.b**) and a significant positive correlation between percent failure and average time to maximum velocity ($r^2= 0.4168$, p -value < 0.01) (**Figure 5.4.c**). These observations suggest that increased efficiency is correlated with a faster motion being adapted over time. Individual animal correlation showed average time to maximum velocity to percent success was a strong predictor of natural variation in percent success (**Figure 5.4.d**). To capture natural variation in motor performance, we analyzed the distribution of time to maximum velocity across each day for successful and failed reaches (**Figure 5.4.e**). Time to maximum velocity for both successful and failed reaches decreased across days, most notably for animals 2, 3 & 4 (**Figure 5.4.e**), which was also observed concomitantly with increased percent success (**Figure 5.4.d**). For animal 1 we did not observe similar patterns, likely due to its relative efficiencies in successful reaching of the pellets, and relatively fast time to maximum velocity compared to other animals. For animal 5 the time to maximum velocity for both successful and failed reaches was large compared to other animals and did not change across days consistent with its inefficiency in pellet reaching.

Mice do not correct the effort of reaching strategies after failure

We plotted the sequences of time to maximum velocity for all attempts during the 10-minute trial across days (**Figure 5.5**). Sequence analysis of time to maximum velocity per reach between trials showed variability in the magnitude of time to maximum velocity for failed reaches (**Figure 5.5, top panel**) and successful reaches (**Figure 5.5, bottom panel**). Unlike peaks (**Figure 5.3**), we did not observe corrective feedback between trials. Peaks in reaching decreased within trials, however, time to maximum velocity did not change within trials, although it was a strong predictor of success. We speculate this highlights a specific role for the timing of distinct neural circuits in shaping specific aspects of the motor output.

Mean squared jerk does not inform motor efficiency in mice

In humans, mean squared jerks of wrist and trunk motions increase in patients with Parkinson's disease (Mancini et al., 2011; Teulings et al., 1997). Post-stroke individuals also exhibit increased mean squared jerk during reaching which is typically associated with a less efficient motion (Hogan & Sternad, 2009). In our study, there was significant increase in mean squared jerk ($p < 0.05$) for successful reaches between D1 and D4 (**Table 5.2**). There was a significant increase ($p < 0.05$) in mean squared jerk for failed reaches between D2 and D4 (**Table 5.2**). Linear regression analysis showed neither significant correlation between mean squared jerk and percent success nor percent failure.

DeepLabCut is optimal for automatic tracking of fine motor reaching

It is well characterized that symptomatic female *Mecp2* heterozygous rodent models have gross motor deficits (Achilly et al., 2021; Bhattacharjee et al., 2017; De Filippis et al., 2015; Morello et al., 2018; Patterson et al., 2016; Samaco et al., 2013). However, it is unclear if pre-symptomatic Het, which do not display overt motor deficits, have underlying fine motor deficits which precede the onset of gross motor deficits. Identification of subtle changes in fine motor function are essential for understanding when and how to apply therapeutic strategies to ameliorate deficits (Achilly et al., 2021). Characterization of learning of fine motor skills over time is needed to address this question, however, a substantial amount of man-power and time goes into manual tracking and often introduces observer bias over extended periods of labeling. Recent advances in computational neuroethology tools, such as DeepLabCut allow for fast and robust tracking of mouse behavior to extract dynamics of behavior in healthy and disease states (Mathis et al., 2018; Nath et al., 2019; Pereira et al., 2022; Wiltischko et al., 2020). To achieve these goals, we thus used DeepLabCut to generate pose and quantified reaching trajectory across fine motor training in the single pellet grasping assay in adult female WT (N=4) and adult pre-symptomatic Het (N=6) mice (**Figure 5.6**).

We trained mice for 17 consecutive days in the single pellet reaching assay and focused on three intermediate time points which represent early phase (Day 3), middle

phase (Day 8) and late phase (Day 14) of training). To confirm mice are motivated to reach for the pellet, we scored their gross motor performance on Day 3, 8 and 14. There were no significant differences in the number of attempts between or within genotype across training (**Figure 5.7.a**). There was no significant difference in percent success of pellet retrieval across training within each genotype (**Figure 5.7.b**). On Day 3 and 8 of training, WT and Het percent success were not significantly different, however, on Day 14 WT performed significantly better than Het at pellet retrieval (**Figure 5.7.b * $p > .05$**). In order to understand differences in motor performance, we plotted example reaching trajectories for WT successful and failed attempts on Day 3 and 14 (**Figure 5.7.c-d'**) and Het successful and failed attempts on Day 3 and 14 (**Figure 5.7.e-f'**). On Day 3 of training, WT and Het have convoluted reaches towards the pellet in failed attempts (**Figure 5.7.c, e**) which are less convoluted in successful pellet retrieval (**Figure 5.7.c', e'**). Compared to Day 3 of training, WT and Het on day 14 of training visually appear to have less convoluted reaches towards the pellet in failed attempts (**Figure 5.6.d, f**) and more efficient straight motions toward the pellet during successful pellet retrieval (**Figure 5.6.d', f'**). This suggest both WT and Het may be adapting more efficient reaching strategies over time.

Het maintain vigor and but not efficiency of reach strategies over training

To quantify efficiency of reaching trajectories, we extracted the mean speed and straightness of the successful and failed attempts across training for WT and Het. Compared to day 3 of training, the mean speed of WT successful reaches significantly increased on Day 8 and significantly decreased on Day 14 (**Figure 5.8.a, black**). Compared to day 3 of training, the mean speed of Het successful reaches significantly increased on Day 8 and 14 (**Figure, 4.8.a, red**). For successful reaches, WT and Het had similar mean speed on Day 3 and 14, however, on Day 8, Het had significantly decreased mean speed compared to WT (**Figure 5.8.a**), suggesting a delay in modulating speed of reaches compared to WT. Compared to day 3 of training, the mean speed of WT failed attempts significantly increased on Day 8 and 14, and plateaued on Day 8 (**Figure 5.8.b, black**). Compared to day 3 of training, the mean speed of Het failed attempts significantly increased on Day 8 and 14, and the mean speed increased between Day 8 and 14 (**Figure 5.8.b, red**). Het failed reaches were significantly

decreased on Day 3 of training but not Day 8 and 14 compared to WT (**Figure 5.8.b**). To determine if mean speed informs gross motor performance, we performed linear correlation analysis between mean speed of successful attempts and % success (**Figure 5.8.c**) and mean speed of failed attempts and % failure (**Figure 5.8.d**) and found no correlation regardless of genotype. This indicates that mean speed does not inform pellet retrieval performance. The findings collectively suggest Het are overall able to adapt changes in speed over time, comparable to WT.

One metric used to measure the smoothness of a two-dimensional motion is the straightness of a motion (**Table 2.1**) (McLean & Skowron et al., 2018). Values closer to one are straighter and more efficient, whereas values closer to zero are considered more random and inefficient (Batschelet, 1981; Benhamou, 2004). Compared to day 3 of training, the straightness of WT successful reaches significantly increased on Day 8 and 14 (**Figure 5.9.a, black**). Compared to day 3 of training, the mean speed of Het successful reaches significantly increased on Day 8 but not Day 14 (**Figure 5.9.a, red**). Between Day 8 and 14, the straightness of successful reaches for Het decreased significantly, suggesting a regression as Het are unable to maintain efficiency which coincides with significantly decreased percent success of pellet retrieval (**Figure 5.7.b**). Interestingly, compared to WT, Het have significantly straighter successful reaches on Day 8 but not 14 (**Figure 5.9.a**).

Compared to day 3 of training, the straightness of WT failed reaches significantly decreased on Day 8 (**Figure 5.9.b**). Between Day 8 and 14 straightness of failed reaches significantly increased back to baseline (**Figure 5.9.b, black**). We speculate the WT shift in decreased straightness is because the mice are prioritizing mean speed of the reach to determine the most efficient strategy to successfully retrieve the pellet (**Figure 5.8.b, black**). Compared to day 3 of training, the straightness of Het failed reaches significantly increased on Day 8 but not Day 14 (**Figure 5.9.b, red**). Between Day 8 and 14, the straightness of failed reaches for Het decreased significantly, suggesting a regression as Het are unable to maintain efficiency of reaching strategies which coincides with significantly decreased percent success of pellet retrieval on Day

14 (**Figure 5.7.b**). Similar to findings in successful reaches, Het have significantly straighter failed reaches on Day 8 but not 14, compared to WT (**Figure 5.9.b**), suggesting Het may overcompensate in reaching strategies due to issues with integrating sensory and motor information. To determine if straightness informs gross motor performance, we performed linear correlation analysis between straightness of successful attempts and % success (**Figure 5.9.c**) and found no correlation regardless of genotype. Linear correlation analysis revealed that straightness index is significantly negatively correlated with % failure for Het ($r = -0.58$, $p\text{-value}=0.022$, red) but not WT (black) (**Figure 5.9.d**), suggesting straightness informs variation in inefficiency in Het.

Since we observed a significant decrease in straightness of reaches from Day 8 to 14 in Het, we performed correlational analysis by day to determine if straightness inform percent success of pellet retrieval. Regardless of day, straightness of successful attempts was not significantly correlated with percent success for WT (**Figure 5.10, black, top panel**). However, the straightness of successful attempts was not correlated on Day 3 and 9 but was significantly positively correlated with percent success on Day 14 (**Figure 5.10, red**). These indicate straightness is an informative metric to measure individual variation in fine motor consolidation in Het.

Discussion

Establishment of human motor kinematics to study fine motor learning in mice

Since the introduction of the single pellet-reaching assay in 1980s, many groups have contributed to our knowledge on the neural circuitry required for fine motor skill execution. Previous studies validated the application of qualitative and quantitative kinematic parameters to assess recovery of motor function in stroke models of rats and mice, however, it is unclear if human kinematics are sensitive to acquisition of the skill (Berthier & Keen, 2006; Braun et al., 2012; Clarke et al., 2007; Lai et al., 2015).

Clinicians assess motor efficiency by using scoring systems combined with kinematic parameters such as peaks, mean squared jerk, and time to maximum velocity

(Bosecker et al., 2010; Carr et al., 1985; Fugl-Meyer et al., 1975). Scoring systems in rodents are not ideal as these systems rely on the presence or absence of motor deficits and cannot discern more subtle changes in movements in rodents (Sindhurakar et al., 2019). Growing evidence from qualitative and quantitative analyses of reach kinematics supports the notion that motions utilized by rodents and humans in reach-to-grasp task are homologous (Becker et al., 2020; Buitrago et al., 2004; Karl & Whishaw, 2013; Klein et al., 2012; Sacrey et al., 2009; Whishaw et al., 1992). Fine details of the reach-to-grasp motion in healthy mice are needed to identify subtle changes induced by motor perturbations, decipher motor learning sequences, and understand how these features are disrupted in motor disorders.

Our findings identify kinematic parameters that are sensitive to subtle changes in movements during fine motor skill acquisition in mice. According to our results, the numbers of peaks is an informative measure of motion efficiency in mice. Peaks of failed reaches decreased with exposure and were comparable to peaks of successful reaches on D4. Peaks of failed reaches were a strong indicator of natural variation in percent success retrieval. We speculate peaks of successful reaches did not change significantly with training due to a “ceiling effect” for maximum biological or mechanical efficiency. In addition, we found time to maximum velocity decreased significantly with exposure and was a strong indicator of natural variation in success.

Furthermore, our results indicate a correlation between increased percent success and percent failure and efficiency of movements with short time to maximum velocity and minimal peaks, respectively. Sequence analysis suggests animals used performance feedback to alter reaching strategies between trials; these alterations were apparent in the number of peaks, but not time to maximum velocity, suggesting distinct neural circuits may shape the speed and efficiency of a motion at different time scales in response to corrective feedback after success and failure, respectively.

Differentiating neural circuits for reaching and grasping during fine motor learning

Dopaminergic circuits in the striatum of mice are important for modulating reach velocity, however it is unclear what circuits modulate sub movements in reaches (Panigrahi et al., 2015). The identity of these circuits may be related to specific neural circuits important for controlling the extrinsic (reaching) and intrinsic (grasping) properties for successful pellet retrieval (Azim et al., 2014; Esposito et al., 2014). One limitation from our study is our analysis focused on applying kinematics to the reaching component of this assay and did not focus on the grasping component. Failed trials could be due to animals not grasping properly in some trials, but these potential instances were not excluded from our analysis. As both the execution of reaching and grasping are important for successful retrieval, future work quantifying natural variation in mouse reaching and grasping kinematics will be required to investigate the underlying neural circuitry and its substrates.

We found that mean squared jerk was not an informative measure of fine motor skill acquisition. Previous studies used mean squared jerk to quantify ballistic motions with set start positions. We reason that mean squared jerk is not an accurate measurement for this assay as free moving mice may start at any variable position and mean squared jerk is not sensitive to this variability (Becker et al., 2020). It is still possible that mean squared jerk may be a strong predictor for the acquisition of fine motor skills in head fixed mice, as the consistent start position mimics smooth target directed movements from previous human studies.

Application of computational neuroethology tools to study fine motor learning

Assays such as rotarod performance test, forelimb hang, single pellet reaching assay are traditionally used to study fine motor learning. Licensed software is available to extract fine motor details from these behaviors; however, the amount of time and resources required for extracting fine details of motor behavior is substantially high. New approaches using deep learning allow for fast, automated, marker-less, pose

estimation of free moving behavior in rodents (Graving et al., 2019; Mathis et al., 2018; Nath et al., 2019; Nilson et al., 2021; Pereira et al., 2019). Mouse movement kinematics such as peaks, time to maximum velocity and more quantitative parameters can be quickly extracted from robust pose estimation data generated by DeepLabCut, DeepPoseKit, SLEAP and SimBA. Our findings provide accessible quantitative measurements that can be used in future studies of fine motor skill acquisition and consolidation in healthy condition and models for neurological diseases.

In conclusion, through manual analysis of individual reaches during fine motor skill acquisition, we show that kinematic parameters identified from human studies such as peaks and time to maximum velocity are strong predictors of improved motor efficiency in adult mice. Our findings highlight kinematics that are sensitive to natural variability in fine motor skill acquisition which could reflect individual changes in the neural circuitry and neural substrates required for motor skill learning. We also validate the application of DeepLabcut for studying fine motor learning in female WT and Het mice. We speculate on the findings from the Het fine motor studies further in the conclusion discussion. This opens future directions to use in-vivo manipulation combined with real-time tracking to investigate the neural mechanisms of fine motor learning in healthy and diseased states.

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Appendix

Table 5.1. Average maximum velocity of failed and successful reaches across days.

Mean $\pm$ S.E.M. of maximum velocity across multiple reaches $n = 16-84$ per day, for 5 mice.

Maximum velocity (mm/s)	D1	D2	D3	D4
Failed	447.81 $\pm$ 24.50	401.88 $\pm$ 20.61	426.43 $\pm$ 18.39	411.20 $\pm$ 13.03
Success	375.83 $\pm$ 26.22	414.89 $\pm$ 26.03	405.83 $\pm$ 19.14	401.80 $\pm$ 17.21

Table 5.2. Average Mean Squared Jerk of failed and successful reaches across days. Mean $\pm$ S.E.M. of mean squared jerk across multiple reaches $n = 16-84$ per day, for 5 mice. Bold values indicate significance with corresponding letter, a = * $P < 0.05$, b = ** $P < 0.01$.

Mean Squared Jerk (mm ² s ⁶)	D1	D2	D3	D4
Failed	3.60x10 ¹³ $\pm$ 2.48x10 ¹²	3.29x10 ¹³ $\pm$ 3.94x10 ¹² ,a	3.72x10 ¹³ $\pm$ 2.12x10 ¹²	4.43x10 ¹³ $\pm$ 2.85x10 ¹² ,a
Success	3.39x10 ¹³ $\pm$ 3.94x10 ¹² ,b	4.19x10 ¹³ $\pm$ 3.12x10 ¹²	4.36x10 ¹³ $\pm$ 3.35x10 ¹²	4.72x10 ¹³ $\pm$ 3.17x10 ¹² ,b

Table 5.3 Number of animals included and excluded from DLC analysis. Het_j was excluded from analysis due to low number of attempts across training.

Animal	Id	Day	Number of successes used in analysis	Number of failed attempts used in analysis	Total number of attempts used in analysis
het_a	5	3	20	22	42
het_f	6	3	12	10	22
het_g	7	3	18	18	36
het_h	8	3	16	16	32
het_j	9	3	6	10	16
het_j	10	3	0	0	0
wt_b	1	3	36	14	50
wt_e	4	3	23	27	50
wt_d	3	3	17	19	36
wt_c	2	3	16	20	36
het_a	5	8	21	21	42
het_f	6	8	19	17	36
het_g	7	8	22	22	44
het_h	8	8	26	24	50
het_j	9	8	24	26	50
het_j	10	8	0	0	0
wt_b	1	8	23	21	40
wt_e	4	8	25	25	40
wt_d	3	8	21	28	46
wt_c	2	8	13	13	26
het_a	5	14	7	9	16
het_f	6	14	17	27	38
het_g	7	14	17	23	28
het_h	8	14	27	23	40
het_j	9	14	14	13	24
het_j	10	14	0	0	0
wt_b	1	14	31	13	38
wt_e	4	14	18	25	40
wt_d	3	14	26	24	50
wt_c	2	14	23	24	44

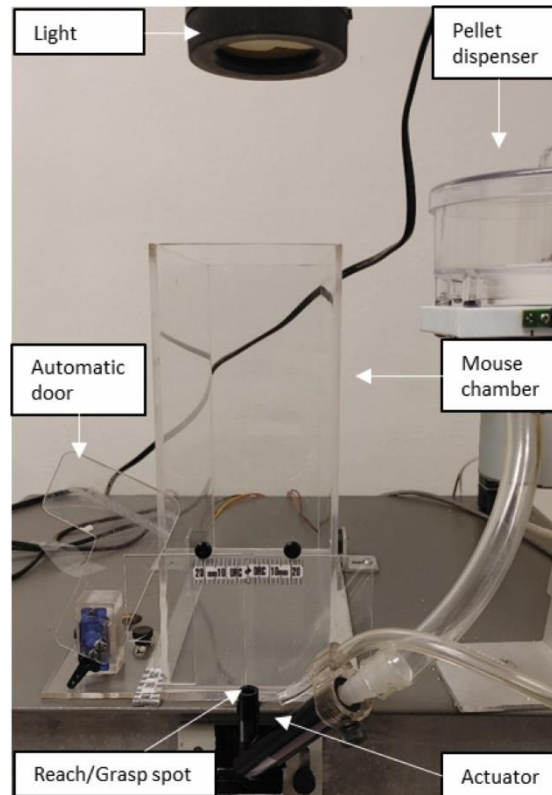


Figure 5.1. Representative picture of in-house motor apparatus. All major components of the motor apparatus are labeled within the diagram.

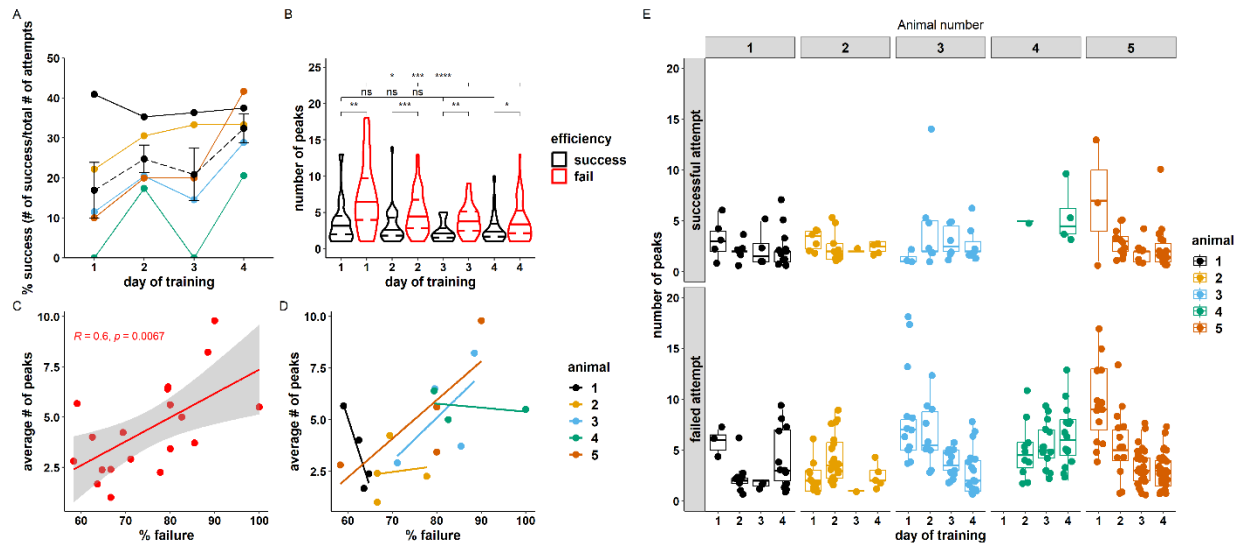


Figure 5.2. Peaks inform individual variation in percent failure measurement.

(a) Average percentage of successful pellet retrieval (number of success/total number of attempts) across training for $n=5$ mice. Mean $\pm$ S.E.M. of the percentage for each day of training (dashed black line). Animals are color coded by their percent success trendlines. (b) Number of peaks per reach across training for both success and failed reaches during the pellet retrieval of 5 mice ($n=378$). Violin plots represents median and distribution of all interactions in reaching for that day, color coded by genotype. Statistical significance determined by non-parametric Kruskal Wallis followed by Dunn's test, * $P<0.05$, ** $P<0.02$, *** $P<0.01$, **** $P<0.001$. (c) Linear regression analysis between the average number of peaks across training and percent failure. Each point represents the average number of peaks for failed reaches of one animal on a specific day. Line of best fit for proposed model and the 95 percent confidence interval is marked by the gray boundary; (r) = 0.5667, Variance (r^2) = 0.3213, p -value = 0.0064. (d) Individual correlation between average number of peaks and percent failure rate for each animal on a given day. (e) Individual boxplots showing the distribution of peaks for each animal on each day across training. Box plots represent the median of each dataset.

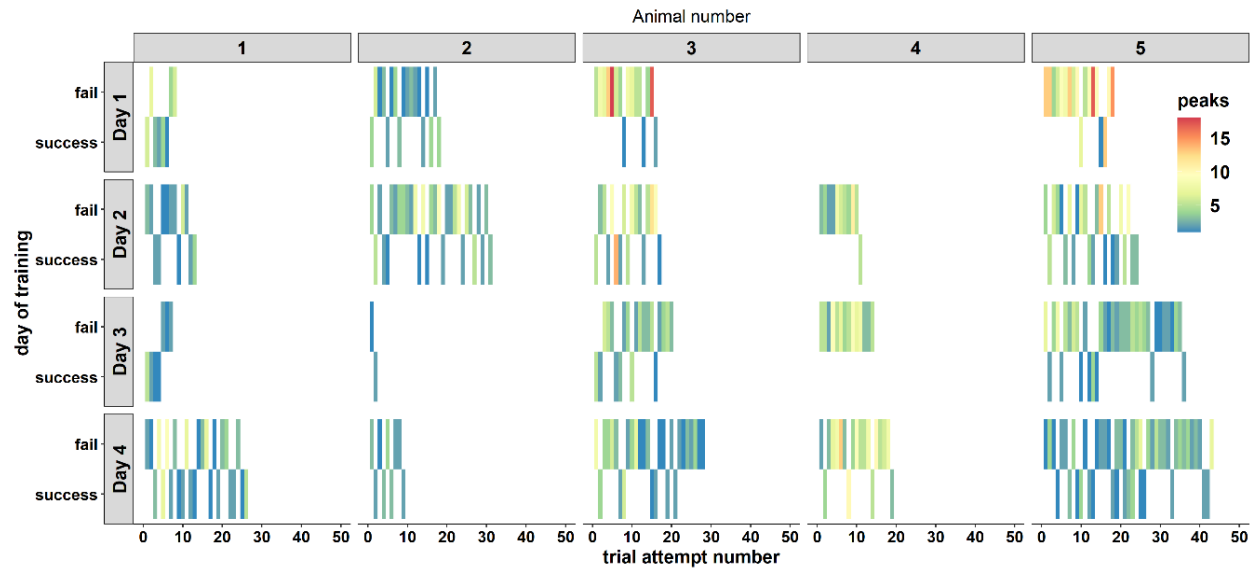


Figure 5.3 Mice correct the number of peaks in their reaching strategies after failure.

Sequence analysis of number of peaks for all reaches during a trial on each day per animal. The number of peaks are denoted by a color scale; warmer colors indicate a larger number of peaks and cooler colors indicate a small number of peaks. Y axis represents the day of training grouped by efficiency (fail or success), and x axis represents the trial number of each reach grouped by animal.

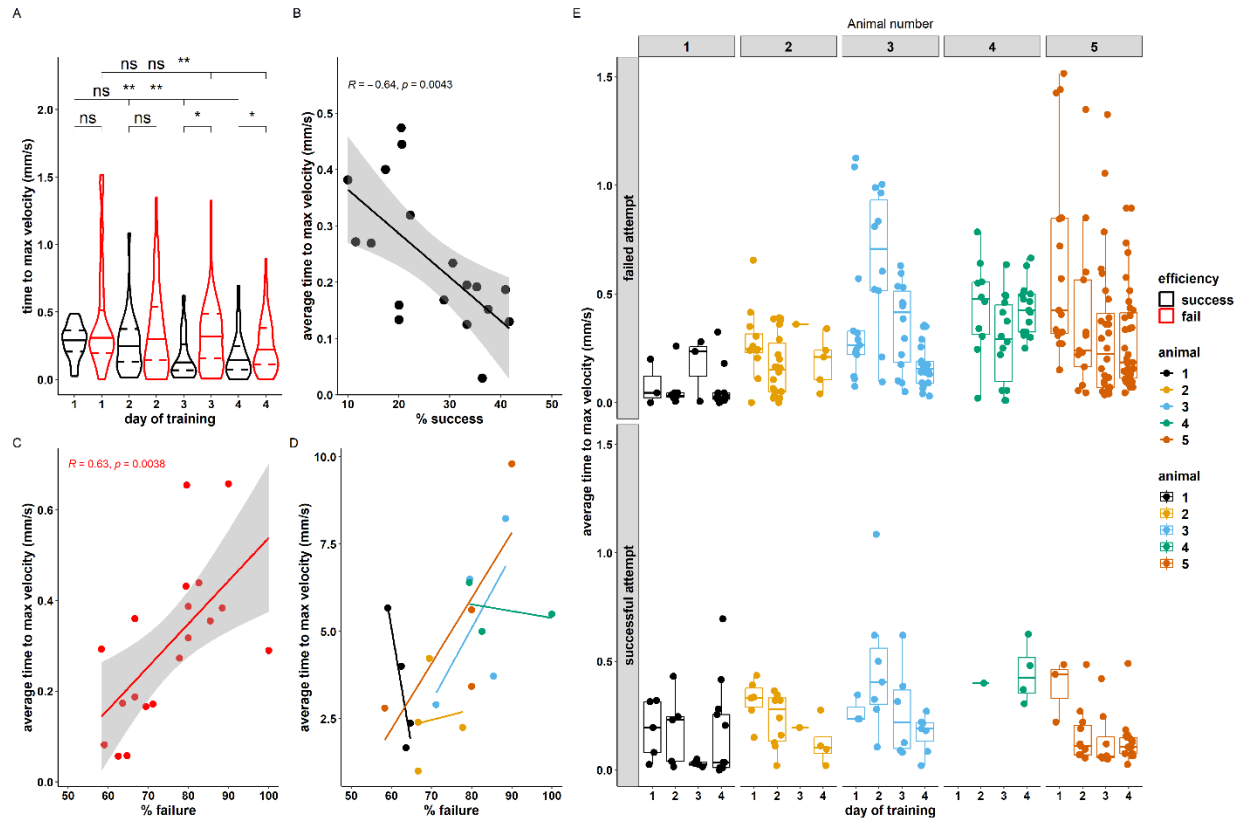


Figure 5.4 time to maximum velocity is an informative measure for individual variation in percent success. (a) Average time to maximum velocity across training for both success and failed reaches during the pellet retrieval of 5 mice (n=378). Violin plots represents median and distribution of all interactions in reaching for that day, color coded by genotype. Statistical significance determined by non-parametric kruskal-wallis followed by dunn's test, *p<0.05, **p<0.02, ***p<0.01, ****p<0.001. **(b, c)** Linear regression analysis between the average time to maximum velocity and percent success. Each dot represents the average time to maximum velocity of one animal on a specific day. Line of best fit for proposed model and the 95 percent confidence interval of the model is marked by the grey bands; for success ($r = 0.6159$, variance (r^2) = 0.3739, p-value = 0.0042). **(d)** Individual correlation between average time to maximum velocity, and percent success rate for each animal. **(e)** Individual boxplots showing the distribution of time to maximum velocity for successful and failed reaches for each animal on each day. Box plots represent the median of each dataset.

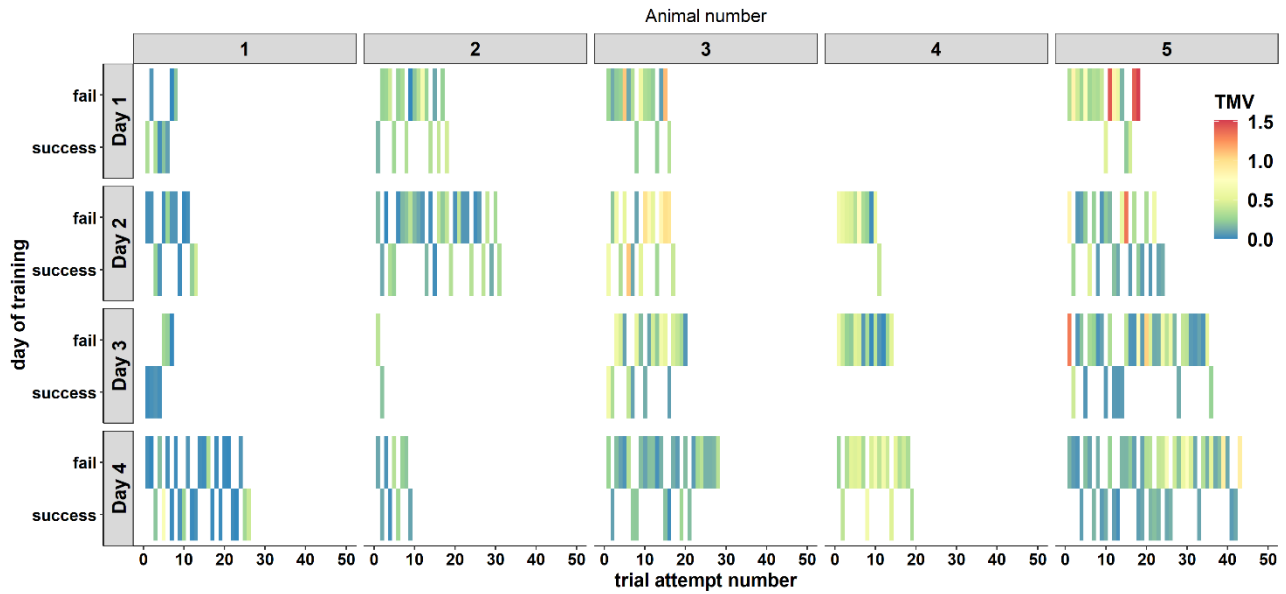


Figure 5.5. Mice do not correct the effort of reaching strategies after failure. Sequence analysis of time to maximum velocity for all reaches during a trial on each day per animal. Time to maximum velocity (TMV) is color scaled (warmer colors indicate a slow time to maximum velocity and cooler colors indicate a fast time to maximum velocity). The y axis represents the day of training grouped by efficiency (fail or success) and x axis represents the trial number of each reach grouped by animal.

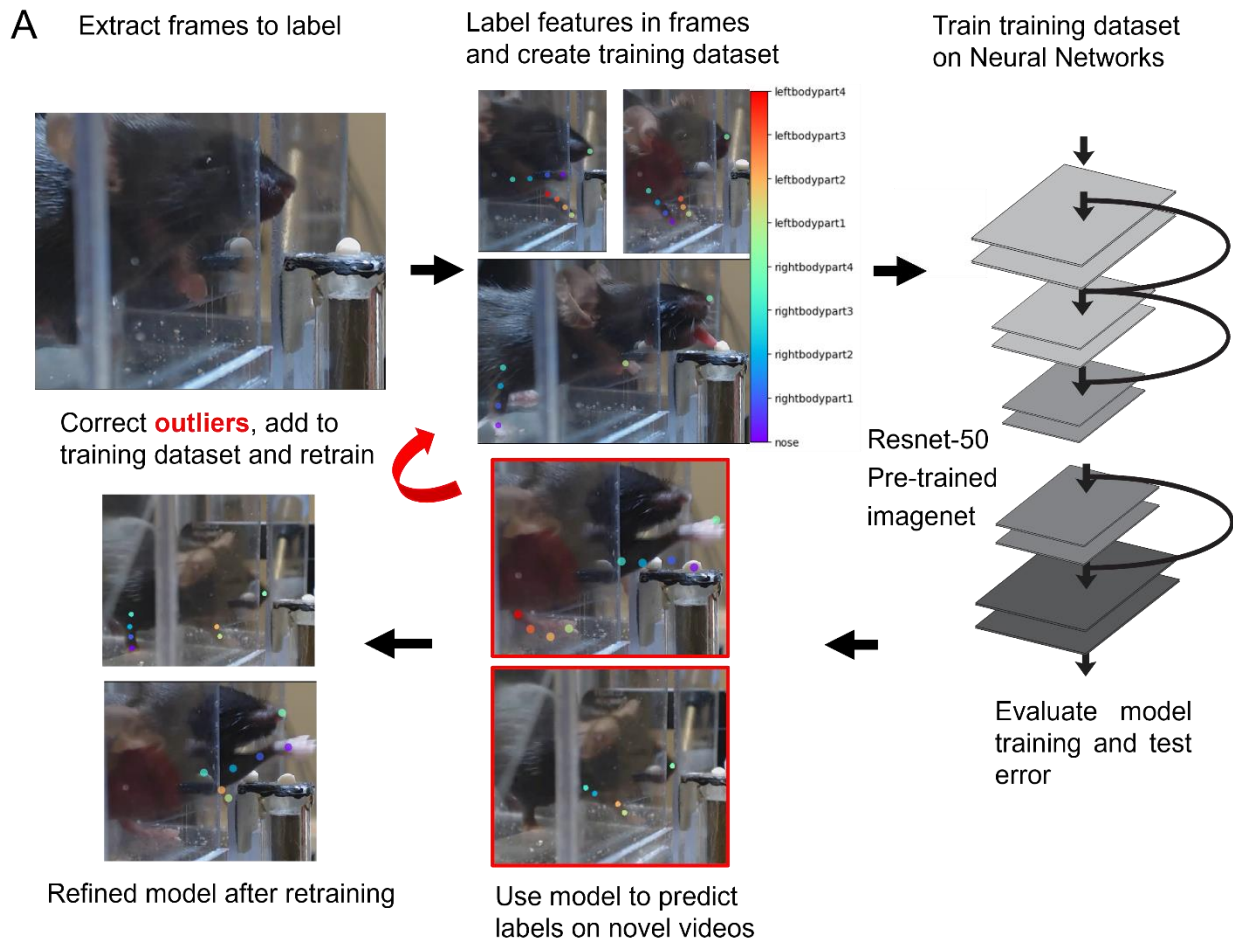


Figure 5.6 DLC derived pipeline for analysis of reaching trajectories.

(a) We trained videos of behavior in single animal DeepLabCut (DLC) to predict pose of the animal. Features of interest were labeled and trained in DLC to build pose models which were then refined with more training to correct for errors. From our models we generate pose estimation and extract features of interest such as the nose to c) analyze trajectories of our mice in R using the Traj package (McLean and Skowron Volponi 2018).

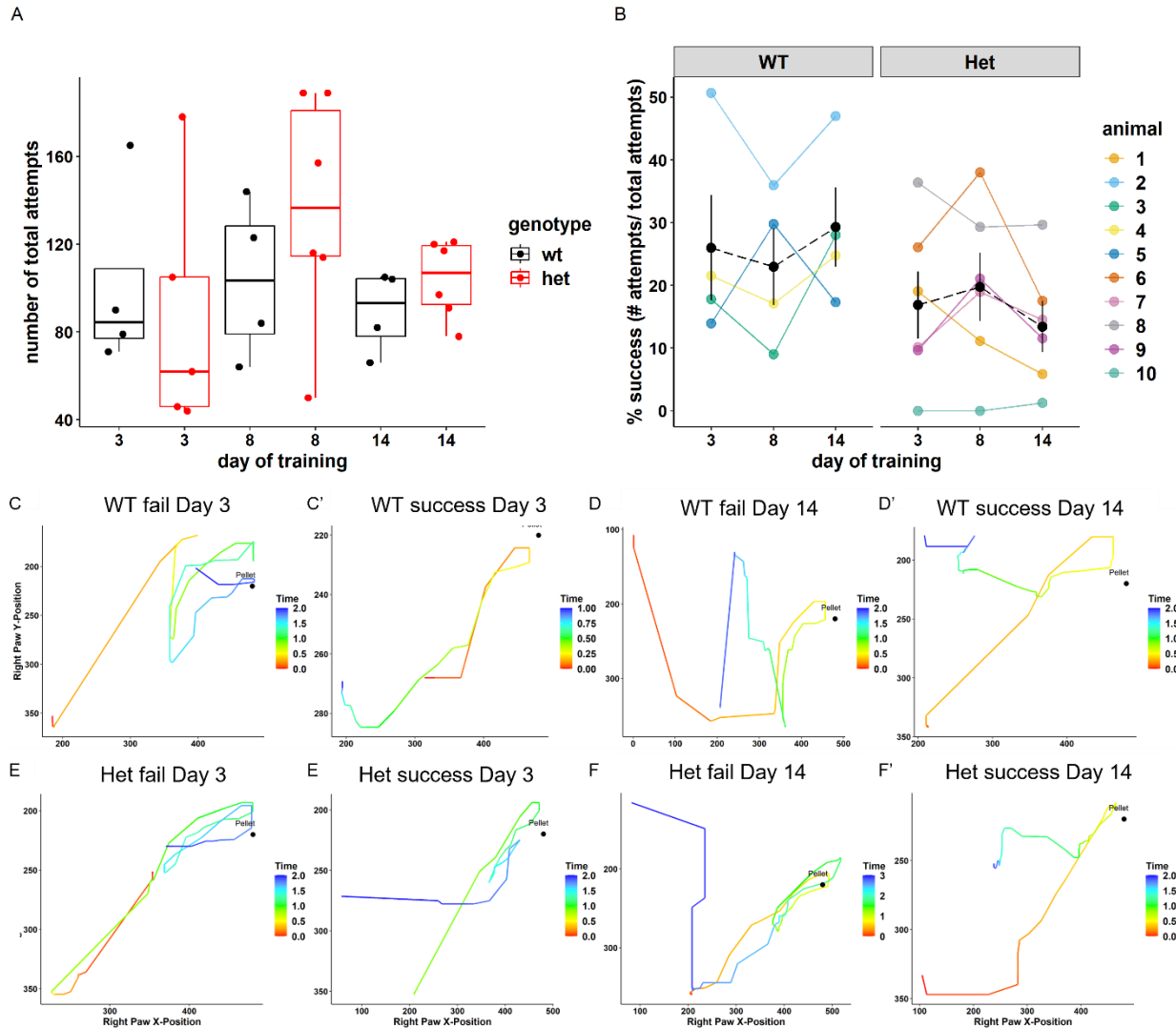


Figure 5.7. Adult Het perform worse than WT in late phase training.

(a) Median number of total attempts on day 3, 8 and 14 of training for each genotype. (WT=black, Het=red). Circles represent an individual animal on that day and are color coded by genotype. Kruskal-Wallis with uncorrected dunn's test revealed no significant differences in the number of attempts between genotype or within genotype across training. **(b)** Average percentage of successful pellet retrieval (number of success/total number of attempts) across training for n=4 WT mice and n=5 Het mice. Mean $\pm$ S.E.M. of the percentage for each day of training (dashed black line). Animals are color coded by their percent success trendlines. Kruskal-Wallis with uncorrected dunn test revealed no difference in % success across training in WT or Het and decreased performance in Het on day 14 compared to WT **(c-f)** Examples of reaching trajectories of the X-Y position of the right wrist relative to the pellet (black dot). for **(c)** WT failed attempt on Day 3, **(c')** WT success on Day 3, **(d)** WT failed attempt on Day 14, **(d')** WT success on Day 14, **(e)** Het failed attempt on Day 3, **(e')** Het success on Day 3, **(f)** Het failed attempt on Day 14, **(f')** Het success on Day 14 of training. Trajectories are color coded by time.

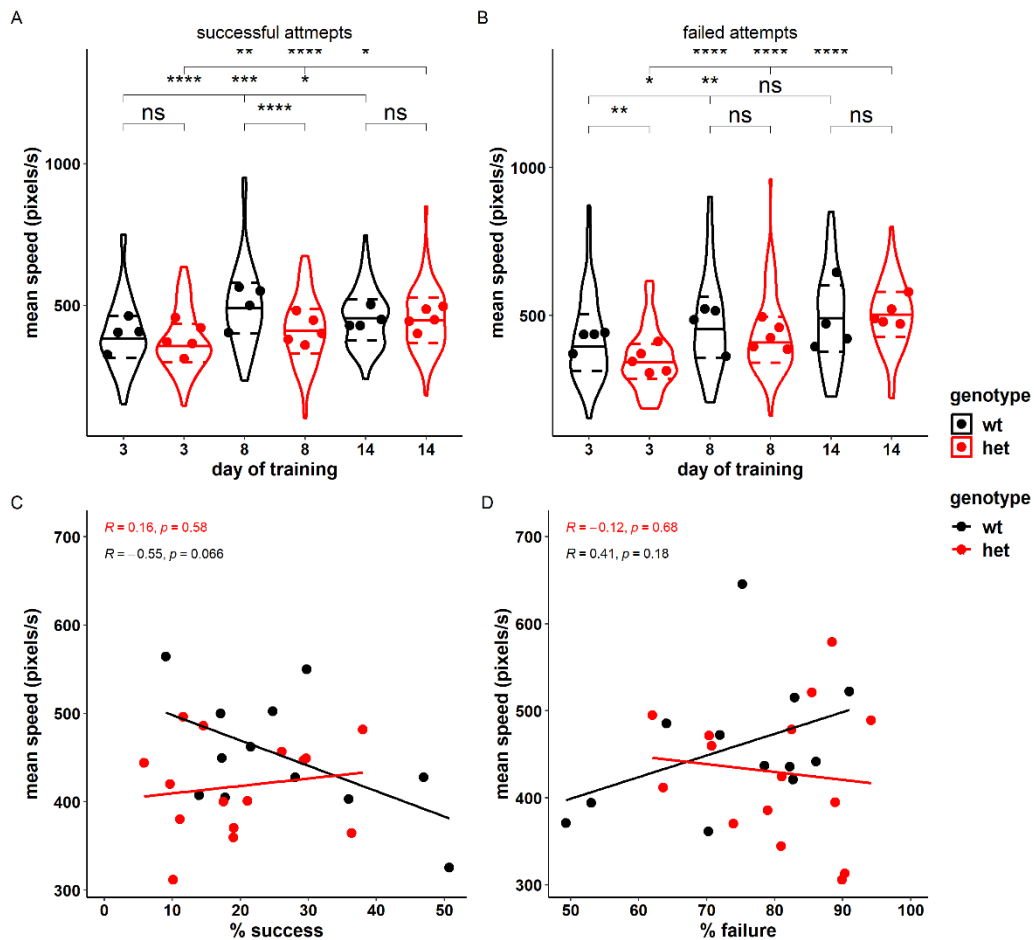


Figure 5.7 Mean speed of reaches increases with training in WT and Het mice

(a) Median mean speed for successful reaches on day 3, 8 and 14 of training for each genotype. (WT=black, Het=red). **(b)** Median mean speed for failed reaches on day 3, 8 and 14 of training for each genotype. (WT=black, Het=red). **(a-b)** Violin plots represents median and distribution of all interactions in reaching for that day, color coded by genotype. Circles represent an individual animal on that day and are color coded by genotype. Statistical significance determined by non-parametric kruskal-wallis followed by dunn's test, * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$, **** $p < 0.001$. **(c-d)** Linear regression analysis between the mean speed across training and percent success, color coded by genotype [WT (black): ($r = 0.55$, Variance (r^2) = 0.3025, p -value = 0.066; Het (red): ($r = 0.16$, Variance (r^2) = 0.0256, p -value = 0.58] and **(d)** mean speed across training and percent fail, color coded by genotype [WT (black): ($r = 0.41$, Variance (r^2) = 0.16, p -value = 0.18; Het (red): ($r = 0.12$, Variance (r^2) = 0.0144, p -value = 0.68]. **(c-d)** Each point represents the mean speed for successful or failed reaches of one animal on a specific day. Line of best fit for proposed model and the 95 percent confidence interval is marked by the gray boundary.

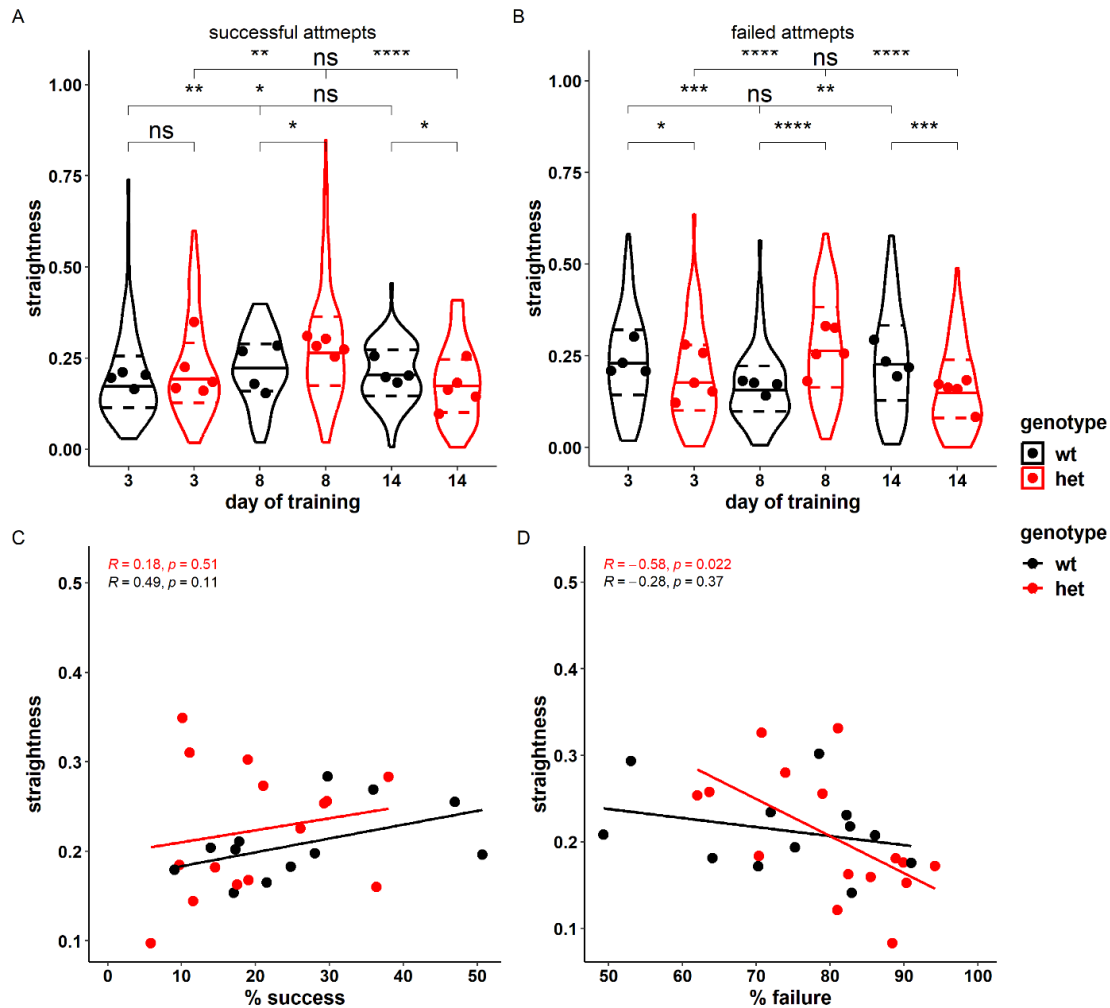


Figure 5.8 Adult Het do not consolidate efficient reaching strategies over time like WT.

(a) Median mean straightness for failed reaches on day 3, 8 and 14 of training for each genotype. (WT=black, Het=red). **(b)** Median mean straightness for failed reaches on day 3, 8 and 14 of training for each genotype. (WT=black, Het=red). **(a-b)** Violin plots represents median and distribution of all interactions in reaching for that day, color coded by genotype. Circles represent an individual animal on that day and are color coded by genotype. Statistical significance determined by non-parametric kruskal-wallis followed by dunn's test, * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$, **** $p < 0.001$. **(c-d)** Linear regression analysis between the mean straightness across training and percent success, color coded by genotype [WT (black): (r)= 0.55, Variance (r^2) = 0.3025, p-value = 0.066; Het (red): (r)= 0.16, Variance (r^2) = 0.0256, p-value = 0.58] and **(d)** mean straightness across training and percent fail, color coded by genotype [WT (black): (r)= 0.41, Variance (r^2) = 0.16, p-value = 0.18; Het (red): (r)= 0.12, Variance (r^2) = 0.0144, p-value = 0.68]. **(c-d)** Each point represents the mean straightness for successful or failed reaches of one animal on a specific day. Line of best fit for proposed model and the 95 percent confidence interval is marked by the gray boundary.

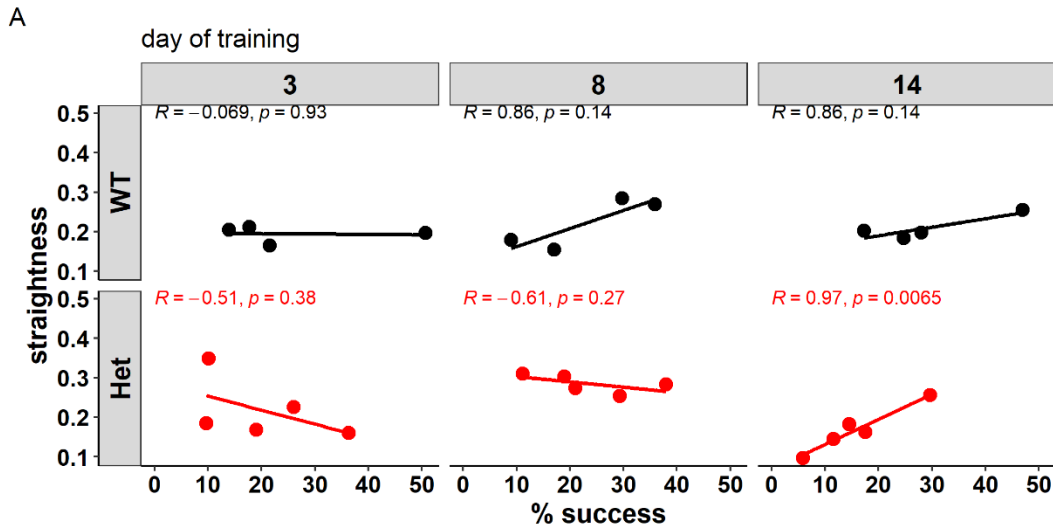


Figure 5.9 Straightness informs individual variation in efficiency in later days of training in Het.

(a) Linear regression analysis between the mean speed of successful reaches and percent success separated by day of training, color coded by genotype [Day 3- WT (black): (r)= -0.069, p-value = 0.93; Het (red): (r)= -0.51, p-value = 0.38 | Day 8- WT (black): (r)= 0.86, p-value =0.14; Het (red): (r)= -0.61, p-value=0.27 | Day 14- WT (black): (r)= 0.86, p-value= 0.14; Het (red): (r)= -0.97, p-value=0.0065].

CHAPTER 6: CONCLUSION

For the past two decades Rett syndrome has been viewed as a neurodevelopmental disorder. However recent research indicates that Rett syndrome is more properly characterized as a neuro-maintenance disorder (Kind and Bird, 2021). Genetic rescue of MECP2 expression in *Mecp2* null male mice reverses neurological symptoms and deletion of MECP2 in WT adult mice is lethal (Guy et al., 2007; McGraw et al., 2011), indicating MECP2 is crucial for the maintenance of the adult mouse brain. These studies opened up the possibility that genetic restoration of MECP2 would have strong therapeutic potential for Rett patients. However random X inactivation in female Rett syndrome patients results in mosaic MECP2 expression, and overexpression of MECP2 leads to MECP2 duplication syndrome which mimics Rett like neurological phenotypes (Collins et al., 2004; Van Esch et al., 2005). Thus, modulating appropriate MECP2 levels is critical for proper neuronal maintenance, providing a challenge to restoring proper MECP2 levels in Rett patients. Here we show that MECP2 expression is age and cell type specific in *Mecp2* Het and coincides with onset of atypical behavior phenotypes and regression in sensory relevant skills. These results indicate dysregulation of wild type MECP2 protein expression as a new disease pathology in Rett syndrome.

MECP2 expression allows for compensation at the behavioral level

We observed significant increases in MECP2 expression between adolescence and adulthood in WT females across cell types (**Figure 1.3.c, f**). Strikingly, adolescent Het non-PV nuclei expressed significantly higher levels of MECP2 than age-matched WT (**Figure 1.3.c, f**), while adult Het non-PV nuclei did not exhibit such a difference (**Figure 1.3.d, g**). Together, these results show that Het displays a precocious increase of wild-type MECP2 levels, as well as an age dependent failure to main wild-type MECP2 expression levels in adulthood (**Figure 1.3.f**), within specific cell types .We speculate the precocious increase of wild-type MECP2 levels in adolescent Het may allow for compensation at the behavioral level (**Figure 1.3, Figure 1.6, Figure 1.7**), and the inability to further increase wild-type MECP2 expression levels on par with WT females leads to behavioral deficits in adulthood (**Figure 1.3, Figure 1.8**) (Mykins et al., 2023b). Although in its early stages, we believe these findings provide a novel path to tackle

neurobiological underpinnings of X-linked neurodevelopmental disorders. The path focuses on the regulation of wild-type protein expression in approximately half of the cells and homeostatic plasticity in the neural networks with non-expressing cells in females over time and age. Ultimately, this observation opens up new avenues to pursue therapeutic strategies to treat Rett syndrome. The impact of these observations will be far-reaching as this well-reported neuropsychiatric disorder typically serves as a model for monogenic, yet complex social and sensory disorders.

Cell autonomous vs non-autonomous regulation of MECP2

Random X-inactivation in Het results in mosaic wild-type MECP2 (MECP2⁺) cells, and cells which do not express functional MECP2 (MECP2⁻). A remaining question in the field is are the phenotypes of MECP2⁺ and MECP2⁻ neurons determined by cell autonomous or non-cell autonomous factors. Previous studies indicate that neuronal MECP2 acts in both a cell autonomous and non-autonomous manner. Knockdown of MECP2 in neuronal culture reduces synaptic scaling and this finding is also observed in vivo in juvenile *Mecp2* null males in which synaptic scaling is abolished in Layer III of the visual cortex (Blackman et al., 2012). Compared to adult Het MECP2⁺ pyramidal neurons, adult Het MECP2⁻ neurons have reduced branching and dendritic length in layer V of the motor cortex (Rietveld et al., 2015), suggesting a cell autonomous role of MECP2. However, compared to WT pyramidal MECP2⁺ neurons, adult Het pyramidal MECP2⁺ neurons have a reduction in soma and nuclei size suggesting a non-autonomous role of MECP2, though it is unclear if this due to the wide age variation (6-21 months) used within the study (Rietveld et al 2015). Alternatively, changes in nuclei size may result from the inability of Het to sustain increased MECP2 expression in non-PV cells over time compared to WT (**Figure 1.3f**).

We speculate MECP2-expressing non-PV cells in Het can respond to external stressors (i.e., mosaicism-induced dysregulation in MECP2⁻ neurons) in a cell-autonomous manner in adolescence. Our results allow for a new hypothesis in Rett syndrome pathology – homeostatic plasticity mechanisms are intact in adolescent Hets, likely owing to the increased MECP2 expression in individual nuclei of non-PV neurons, which

allows for compensation at the molecular, circuit, and behavioral levels. In contrast a lack of maintenance of MECP2 expression over time then leads to disorder and symptomology. The mechanism controlling MECP2 protein expression and degradation is a new direction in the field and is currently unknown.

Correlation between MECP2 expression and PNN expression

We observe that S1BF MECP2 expression in non-pyramidal cells decreases in adult Het and is correlated with increase in PNN expression in adult Het though it is unclear if the two are directly or indirectly related (**Figure 1.3f**) (Billy Y. B. Lau et al., 2020; Mykins et al., 2023b). Recent work suggest that PV+ neuron activity autonomously regulates perineuronal net expression around PV+ cells. Chemogenetically silencing PV+ cells reduces the density perineuronal nets around the silenced PV cells but does not affect neighboring PV+ cell PNN density (Devienne et al., 2021). PV+ cells express extracellular matrix enzymes and proteins which are important for degrading and synthesizing perineuronal net density in response to activity levels. Previously, we reported MECP2 is important for appropriate expression of S1BF PNNs (Billy Y. B. Lau et al., 2020) though the direct relationship between the two is unclear. Loss of MECP2 in PV+ neurons may affect cell autonomous regulations of PNNs and have non-autonomous effects on the activity of neighboring non-PV and PV+ cells. Nearest neighbor analysis of MECP2 expression in surrounding PNN+ and PNN- cells in a cell type specific manner would provide insight on how mosaic expression of MECP2 in PV neurons affects PNN expression.

Identifying difficult to characterize phenotypes in neuropsychiatric disorders

A long-standing problem in the field of neuropsychiatric disorders is the reliable identification of heterogeneous endophenotypes in animal models for the disorders. This problem has clear implications in identifying etiology and therapeutic targets. The emergence of accessible computational neuroethology tools is a powerful solution in systematic characterization of animal behaviors in resolving this heterogeneity. Using

DeepLabCut and multidimensional analysis of an ethologically relevant social cognition task, we identify two distinct populations: one exhibiting efficient behavior and the other displaying an age **(Figure 3.6)** and context dependent **(Figure 3.4)** regression in a female mouse model for Rett syndrome **(Figure 3.3)** (Mykins et al., 2023a). Thus, we have identified a unique sub-population in which to study the cellular substrates of regression during a specific time in the female Het. This is a novel result of significance to Rett syndrome research, as behavioral regression, a key feature of Rett syndrome and other neurodevelopmental disorders has been difficult to model in preclinical animal studies. Additionally, the novel identification of two populations in genotypically-identical mice suggest differential effects on neural circuitry and opens new directions to investigate the underlying molecular and cellular mechanisms and better design individualized experimental therapeutics.

One of the major findings from our work is that we can differentiate individual heterogeneity in our mice solely from behavioral performance. We observed a sub-population of Het non-regressors which improve at pup retrieval over time, albeit, not as well as WT **(Figure 3.3 Het-NR)**, and a distinct population of Het-regressors which improve in early days and regress in later days of pup retrieval **(Figure 3.3, Het-R)**. Prior to behavior, adult Het have increased expression of S1BF PNNs compared to WT, suggesting increased PNNs may inhibit tactile sensory information, ultimately leading to regression (Billy Y. B. Lau et al., 2020). We hypothesized appropriate expression of S1BF PNNs is required for integrating tactile information from pups for efficient retrieval. However, pharmacological degradation of S1BF PNNs in WT did not affect behavior **(Figure 3.2, 3.3)**, and pharmacological degradation of excess PNNs in Het did not rescue behavior **(Figure 3.5, 3.6, 3.7)**. Collectively, these findings suggest abnormal PNN in the S1BF of adult Het is correlative, and not causative of behavioral deficits. Previously, we reported that the % of cells expressing MECP2 in the auditory cortex of adult Het is positively correlated with increased efficiency in pup retrieval behavior on D5 of the behavior (Krishnan et al., 2017), and a precocious increase in MECP2 expression in adolescent allows for behavioral compensation **(Figure 1.3, 1.5, 1.7)** (Mykins et al., 2023b). Based on these findings, we hypothesize that the difference

between these two sub-populations is potentially due to difference in % of cells expressing MECP2, or the expression of MECP2 itself. Future histology studies examining MECP2 expression within these two identified sub-populations will be critical for understanding the differential effects on neural circuitry that allow for compensation in Het non-regressors and maladaptive plasticity in Het regressors.

Potential for therapeutic application

While Het-regressors improve over time like WT and Het-NR in early days (**Figure 3.3**), PCA analysis of early days of retrieval reveals high accuracy in identifying future regressors (**Figure 3.5**), indicating we have identified a critical time point to intervene and ameliorate or prevent regression within this subpopulation (Mykins et al., 2023a). Future directions include time drugged delivery studies to test the efficacy of therapeutics on restoring function within Het regressors. Several therapeutic targets aimed at restoring neurological function in animal models are currently available. Positive allosteric modulators of metabotropic glutamate receptor 7 (mGluR7) agonists show great promise in animal models for Rett syndrome. Rett patients and mouse models for Rett syndrome show decreased expression of mGluR7 in key brain regions related to neurological phenotypes (Gogliotti et al., 2017, 2016). Systemic injection of positive allosteric modulators of mGluR7 receptors rescues contextual fear learning, novel object recognition, and social memory deficits in *Mecp2* null male and *Mecp2* Het female mice (Gogliotti et al., 2017; Jalan-Sakrikar et al., 2014). These drugs are readily available and can be applied to test their effects on preventing potential regression in Het non-regressors or ameliorating regression in Het regressors.

Investigating fine motor learning in mice

The cellular mechanism of how mice learn gross stereotyped and reproducible motor movements that reliably achieve a behavioral goal is unclear. Head-fixed mice learn reproducible movements during a lever water port reaching task and these reproducible movements coincide with specific activity of ensembles in Layer 2/3 of the primary motor cortex (Peters et al., 2014). Optogenetic stimulation of ~ 20 Layer 2/3 pyramidal

neurons (representing the activity ensemble) is sufficient to induce variability in learned movements that are similar to voluntary stereotypical lever push movements (unpublished work by Takaki Komiyama, reviewed here: (Mykins et al., 2023c)). These findings support that specific activity dependent changes in Layer 2/3 pyramidal neurons in the contralateral motor cortex instruct learned stereotypical movements over time (Peters et al., 2017, 2014; Xu et al., 2009). However, how neural mechanisms shape variability in fine motor output into increased efficiency in fine motor skills is unknown.

Here we identified key kinematic parameters that inform learning of efficient motions over time in adult female WT mice. We observed that the number of peaks and time to maximum velocity, and straightness were strong indicators of individual variation in failure and success, during a single pellet reach and grasping assay (**Figure 5.1, 5.4, 5.8, 5.9**) (Mykins et al., 2021). Overall, we validated established human motor kinematics to inform fine motor skill acquisition and natural variation in motion efficiency in mice. Future studies using *In vivo* calcium imaging of established motor neuron ensembles (Peters et al., 2017, 2014) combined with in real time tracking of reaching will allow for further investigation into how neural ensembles are shaped during fine motor learning and perturb in disease states.

Potential fine motor skill consolidation deficits in Het

Our findings indicate that WT mice may prioritize adapting the mean speed and then straightness of their motions in middle phase training (**Figure 5.7, 5.8**) in order to increase percent success of pellet retrieval. In contrast, Het may prioritize straightness and then mean speed of their motions in middle phase training (**Figure 5.7, 5.8**), resulting in an inability to consolidate efficient fine motor skills, and consequently regression in percent success of retrieval (**Figure 5.6**). We speculate this may be due to an accelerated maturation of motor cortical circuits as MECP2 is known to regulate the timing of experience dependent plasticity in the cortex (Fagiolini et al., 2020; Krishnan et al., 2017, 2015; Billy Y.B. Lau et al., 2020; Morello et al., 2018; Patrizi et al., 2020). Loss of MECP2 in Layer 2/3 motor cortex reduces local excitatory synaptic local excitatory

input in Layer 2/3 of the motor cortex of *Mecp2* null males (Morello et al., 2018; Wood et al., 2009; Wood and Shepherd, 2010). This suggest that loss of MECP2 may inhibit or reduce activity dependent changes needed to consolidate stereotypical motions, though it is unclear how heterozygous loss of MECP2 in Het affects motor circuitry.

An important note is that our study does not consider the potential for grasping deficits which may also result in impaired pellet retrieval. Case studies characterize Rett patients as having stereotypical reaching motions but issues with grasping (Buchanan et al., 2019; Stallworth et al., 2019). It is a generally accepted view that Rett patients have grasping deficits, however no clinical studies have quantified the motor mechanics of these deficits. Select studies have identified grasping deficits in symptomatic Het rodent models (De Filippis et al., 2015; Veeraragavan et al., 2016), though neither of these studies followed up with these findings. It is also unclear whether these reported grasping deficits are present in pre-symptomatic Het. Future studies analyzing the neural circuits for grasping (Azim et al., 2014; Bui et al., 2013; Esposito et al., 2014) in pre-symptomatic Het are essential to delineating whether grasping deficits contribute or are the cause of fine motor deficits in Het.

Summary

Establishment of contextually relevant behavioral phenotypes and the associated cellular change in the brain is critical for understanding the underlying pathogenesis of Rett over time. Using a pre-symptomatic *Mecp2*^{heterozygous} female mouse model for Rett syndrome, we find that regression in social and tactile sensory relevant tasks is age dependent and coincides with increased expression of perineuronal nets and the inability to maintain wild-type MECP2 protein expression in the sensory cortex during adulthood (Mykins et al., 2023b). Pharmacological manipulation of perineuronal nets did not rescue regression in social and tactile sensory relevant task, indicating increased PNNs are an indirect consequence of Rett pathology, potential due to the inability to maintain MECP2 expression into adulthood. This allows for futures studies to investigate the role of MECP2 protein expression in the maturation of adult brain and in regulating experience dependent plasticity during ethologically relevant contexts. Through systematic multidimensional analysis of an ethologically relevant behavior, we

identified two sub population of adult Het: Het that display a delay in efficiency days and then improve over days like wild-type mice, and Het that regress and perform worse in later days (Mykins et al., 2023a). This regression is age dependent and suggest differential effects on neural circuitry that allow for compensation or maladaptive plasticity and opens new directions to investigate the underlying cellular and molecular mechanisms in order to better design experimental therapeutics. Additionally, we show potential fine motor deficits in adult Het which coincide during the same time at which regression in sensory skills start to emerge. Our findings collectively provide growing support to the emerging hypothesis that Rett Syndrome is a sensory processing disorder, which ultimately results in progressive regression in social communication, cognition, and motor phenotypes over time.

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APPENDIX

Methods Protocols:

Immunohistochemistry

Brains in Chapter 1 were incubated for 12 hours in a 30% sucrose/PBS solution before sectioning on the freezing microtome. Brains were sectioned at 100 microns in a coronal orientation. Sectioning was done either the day before or day of the immunohistochemistry procedure.

Day 1:

1. Wash sections with 1X PBS, 3 X 5 min
2. Block with 10% normal goat serum + 0.5% Triton-X in 1X PBS, 3 hours
3. Incubate with antibodies (diluted in 1:1000 in 5% normal goat serum + 0.25% Triton in 1X PBS) overnight
4. Store remaining blocking solution at 4°C

Day 2:

1. Light-sensitive, perform under aluminum foiled covered plates
2. Wash sections in 1X PBS, 3 x 5 min
3. Incubate with 2° antibodies (diluted in 5% NGS + 0.25% Triton in 1X PBS), 4 hours
4. Wash sections with 1X PBS for 3 X 5 min
5. Incubate with DAPI solution for 5 min (1 uL of 5 mg/mL in 1 mL 1X PBS)
6. PBS wash 3 x 5 min 1X PBS wash
7. Mount tissues onto slide, and air-dry overnight
8. On the next day, coverslip with Fluoromount-G and seal with nail polish

Mouse behavior protocols

Maternal Behavior

1. Behavior Set Up

- a. Pair a female and male CBA/CaJ together in a large breeding cage
- b. Remove male from cage 1-2 weeks after pairing (or when female is visibly pregnant)
- c. Based on level of pregnancy (judge on size and 21 days after pairing)
 - i. Mother will start to show bulging from the sides and carry weight more caudal
- d. Place a WT and Het female littermate in the cage with the pregnant mother
 - i. For 6WO adolescent behavior, place between the ages of 5-5.5 weeks so that they are ~6 weeks old at the time of postnatal Day 0 (D0)
 - ii. For 12WO adult behavior, place between 11-11.5 weeks so that they ~12 weeks old at the time of postnatal Day (D0)
- e. D0 is the first day of the pup gathering paradigm.
- f. The paradigm is performed on D0, D1, D2, D3, D4 and D5 and the mice are sacrificed and perfused on D5 immediately after behavior.
- g. Behavior is conducted in the home cage, which is placed inside a sound-proof dark box and recorded using a 30 fps infrared camera placed above the cage.
- h. After pairing WT and Het littermates, check every day to see if the mother has given birth to pups

2. Set up and sterilization

- a. Before handling mice: wipe down all stations with MB-10 (desk, keyboard, mouse, outside of soundproof box, and covering of soundproof box)
- b. Before you bring animal cage(s) into room:**
 - i. Run a test video recording session for about 3 minutes, by pointing the camera at a moving object/screen (i.e., play a video on the computer and record this monitor screen).

- ii. Then check for video lags. Proceed if no lags. If lagging, make sure all connections are secure. Turn off everyone and restart.
Repeat step 1. Contact Billy for help if problem persists.
- c. Pup Retrieval Behavior Procedure (perform behavior between 9am - 6pm)
- d. Turn on computer and camera
- e. Check the Avisoft UltraSoundGate: make sure all dials for each channel = 12 o'clock)
- f. Open 'Avisoft RECORDER USGH'
- g. Shortcut folder to recorded audio files is on desktop
- h. Make sure network cable from router is plugged into computer (this is the cable which connects to the camera to record videos)
- i. Click 'SearchTool' on desktop
- j. Click 'R2' or 'R2-2' (or copy and paste address into Internet Explorer)
Make sure you use Internet Explorer!!
 - i. NOTE about IE (if needed): turn off ALL security options under internet options
 - ii. login username krishnanlab, password krishnanlab2
 - iii. make sure time in camera is correct! If not, 'settings', 'basic settings', 'camera time', uncheck 'Sync with NTP server', click 'Sync with PC/Terminal', click 'save'
 - iv. saving file location: 'settings', 'record', 'storage location', 'local recording location' = D:\video (shortcut folder should be on desktop)
- k. Position home cage into box as horizontal as possible, confirm it is centered with the camera by checking the video on the computer.
- l. Check camera settings:
 - i. Mode = 60Hz
 - ii. Stream = user-defined (see below)
 - iii. WDR = off
 - iv. NAA = ON
 - v. IR LED Lights mode = manual - ON

vi. Color Adjustment:

1. 1st = 55
2. 2nd = 59
3. 3rd = 53
4. 4th = 20
5. 5th = 24

m. Mainstream video settings: VIDEO-->VIDEO SETTINGS

- i. resolution = 1080p
- ii. bit rate = 4M
- iii. frame rate = 15
- iv. key frame interval = 30
- v. rate control mode = CBR

n. Sub stream video settings:

- i. stream type = HD mode
- ii. resolution = 720p
- iii. bit rate = 512K
- iv. frame rate = 11
- v. key frame interval = 15
- vi. rate control mode = cbr

o. To record video: click on the camera screen of interest, then click 'record' (bottom right corner, middle icon)

p. To record audio, open Avisoft Recorder USGH (icon on desktop or C:\Program Files (x86)\Avisoft Bioacoustics\RECORDER USGH), click 'red' button on top left corner

q. Audio settings:

- i. each mic is 7 inches away from base of bedding; Gate: 12 o'clock on dial for all channels
- ii. Make sure the cage top that is holding all 4 mics has the 'front' label facing you!
- iii. From computer screen:

1. bottom right = mic #1
 2. bottom left = mic #2
 3. top left = mic #3
 4. top right = mic #4
- iv. Options-->Configuration: Make sure you see all active (X) channels in Device screen
 - v. Trigger = permanent (unlimited), Sampling rate = 375000, Buffer = 0.032s, Format = 16bit, Display = spectrogram, Range: 0-250kHz, FFT size = 256, max time = 30 min, check show time code
 - vi. Filenames: File name format =
T'year"month"date"hour"minute"second'
 - vii. make sure 'pause' button is not selected! time on top right corner should count down when the recording is session
3. Pup retrieval behavioral paradigm
 - a. All adults are removed from home cage and placed in large holding cage for the duration of the behavior
 - b. Behavior is performed with a max of 5 pups. If there are more than 5 pups, the extra pups are placed in holding cage with the adults
 - c. On the first day, randomly select either the WT or Het. For each subsequent day, alternate the order so that the mice don't develop a habit of expecting the behavior.
 - d. Habituation Phase (5 minutes total)
 - i. Place 3-5 pups in the nest and the first selected animal is placed into the home cage. Before placing the animal in the cage follow these instructions.
 1. Grab the mouse by the tip of its tail and hold it on top of the grate of the cage. Quickly press record on the audio and then the video recording software and place the mouse in the home cage which is in the darkbox. Close and lock the door and make mental notes of the time (located on the

video camera recording window) when the door shuts. You will record this as your start time for this animal.

2. After 5 minutes begin isolation phase

e. Isolation Phase (2 minutes)

- i. Remove the pups from the home cage.
- ii. After two minutes begin retrieval phase.

f. Retrieval Phase (5 minutes)

- i. Pups are scattered around the cage in the 3 corners, then center of cage (if 4 pups), then nest (if 5 pups)
- ii. Place initial pups in the locations farthest from the adult that is in the home cage
- iii. At the end of the retrieval phase, the adult is removed from the home cage and placed back in the holding cage
- iv. At five minutes stop audio recording and the video recording
 1. After each recorded animal, view the video and check for lagging (observe ~10 sec at 3-4 different timepoints)
 2. If lagging exist, put all animals back into home cage and return to mouse room. Shut down everything. Wait for 10 seconds and restart everything. Do the test video run as stated at the very top! Repeat shutdown if needed.
 3. For each animal, DO NOT repeat the behavior if she has already gone through the behavior on the same day!

g. For saving Notepad file:

- i. For P0: open 'Maternal short-term Template' notepad file, rename with cohort#_condition_P0 (or genotype_P0,
- ii. save into P0 folder of the new cohort folder
- iii. For next P Day: open previous day (i.e., P0) notepad file, save as cohort#_condition or genotype_Pnewday into P new day folder, and continue with adding new information
- iv. repeat step 2 with previous day notepad file.

- v. At the end of P5, there should be 1 notepad file for each behavior day, with P5 notepad file containing information for all days!
- h. Repeat steps 3d.1 - 3g for the other littermate.
- i. Once all WT and Het have been tested, all animals are placed back in the home cage.
- j. Repeat step 2 & 3 for each subsequent day of behavior until P5

Texture discrimination task

For Chapter 1:

1. Set Up and sterilization
 - a. Behavior box is placed in the sound-proof dark box (specifications of the box and inserts are in Chapter 1 description)
 - b. All behaviors were recorded from the top and bottom view using 30 fps cameras
 - c. Behavior for adolescent animals starts at 5.5-6 weeks old and behavior for adult animals starts at 11.5-12 weeks old
 - d. Perfusions are within 4 hours of completing the discriminations task
 - e. Alternate the order in which the animals are placed into the behaviors each day
 - f. Sterilize the working area with MB-10
2. Habituation (Day 1 & 2)
 - a. Subject is placed in soundproof dark behavior chamber for 10 minutes
 - b. Activity is recorded via video camera
 - c. Subject is removed and box is disinfected with MB-10 to eliminate smells between animals
 - d. Allow 5 minutes between each animal for the box to air out
3. Texture Discrimination (Day 3 & 4)
 - a. Learning/Familiarization Phase (10 minutes)
 - i. Place two identical sandpaper textures (coarse) around the inserts, then place the wrapped insert into the inner holes in the behavior chamber.

- ii. Press record on the camera and place subject in the behavior chamber and shut the door
 - 1. Note the time on the camera when you shut the door, this will serve as your start time
 - iii. Stop recording after 10 minutes from when dark box door was closed
 - b. Retention Phase (5 minutes)
 - i. Remove subject from chamber and place into the home cage
 - ii. Remove inserts and replace with a NEW identical coarse texture as well as a novel texture (Day 3: medium grain; Day 4: fine grain)
 - c. Testing Phase (10 minutes)
 - i. Start recording and indicate the location of the novel texture (hover hand over novel)
 - ii. Place subject in behavior chamber
 - iii. Stop recording after 10 minutes (of the dark box door being closed)
 - iv. Return subject to home cage
 - v. Clean behavior chamber and textures
 - 1. Mist textures with MB-10 and allow to air dry
 - 2. Clean x 2 with MB-10 and allow to air dry for 5 minutes
- 4. Novel Object Recognition (Day 5)
 - a. Learning/Familiarization Phase (10 minutes)
 - i. Place two identical shapes (cubes) into the outer holes in the behavior chamber
 - ii. Press record on the camera and place subject in the behavior chamber and shut the door
 - iii. Stop recording after 10 minutes from when dark box door was closed
 - b. Retention Phase (5 minutes)
 - i. Remove subject from chamber and place into the home cage
 - ii. Remove inserts and replace with a NEW cube as well as novel sphere

- c. Testing Phase (10 minutes)
 - i. Press record on the camera and place subject in the behavior chamber, indicate the location of the novel object, and shut the door
 - ii. Stop recording after 10 minutes from when dark box door was closed
 - iii. Return subject to home cage
 - iv. Clean behavior chamber and objects
 - 1. Mist objects with MB-10 and allow to air dry
 - 2. Clean x 2 with MB-10 and allow to air dry for 5 minutes

Maternal and texture battery test

- 1. Set up and perform maternal behavior as described in the maternal Behavior protocol, with the addition of texture discrimination task on D4 and D5 of maternal behavior
- 2. Set Up and sterilization for texture discrimination task
 - a. Behavior box is placed in the sound-proof dark box (specifications of the box and inserts are in Chapter 3 description)
 - b. All behaviors were recorded from the top and bottom view using 30 fps cameras
 - c. Behavior for adult animals starts at 11.5-12 weeks old
 - d. Perfusions are within 2 hours of completing the discriminations task
 - e. Alternate the order in which the animals are placed into the behaviors each day
 - f. Sterilize the working area with MB-10
- 3. Habituation (Day 4 of maternal behavior)
 - a. Performed after performing D4 maternal behavior for both animals
 - b. Subject is placed in soundproof dark behavior chamber for 10 minutes
 - c. Activity is recorded via video camera
 - d. Subject is removed and box is disinfected with MB-10 to eliminate smells between animals
 - e. Allow 5 minutes between each animal for the box to air out

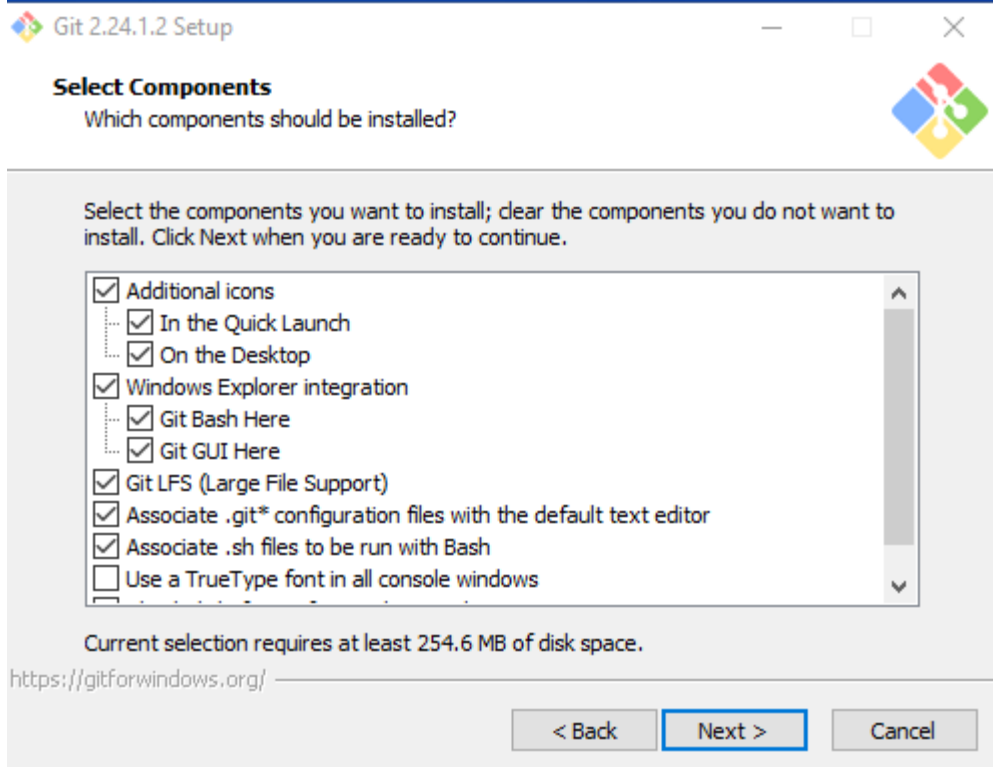
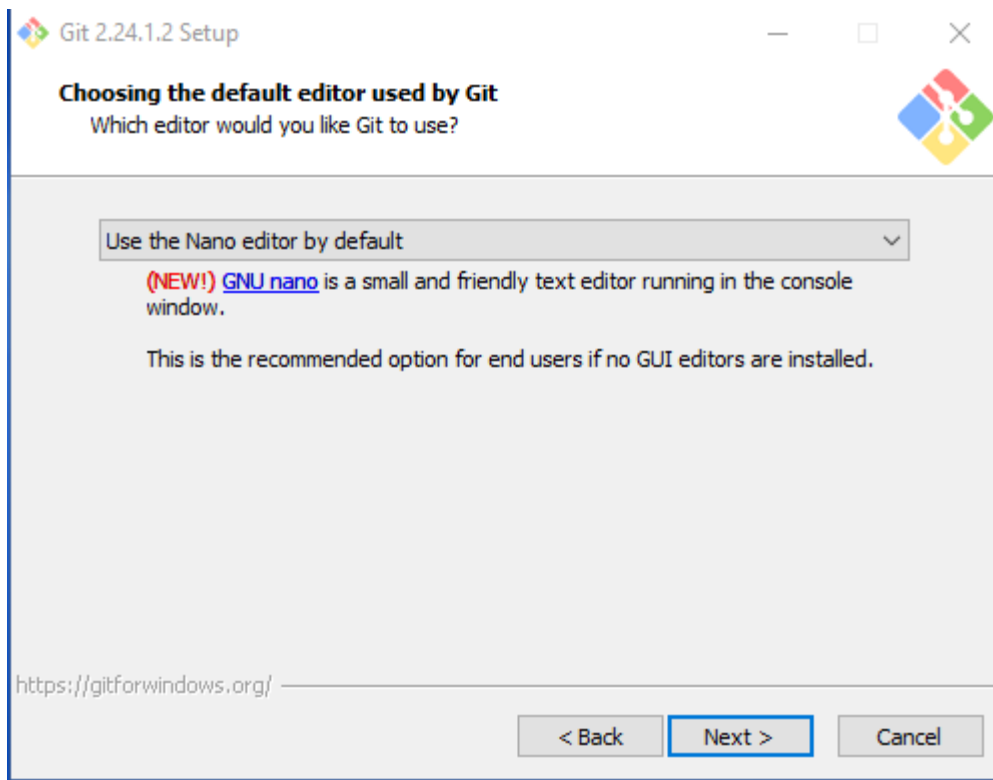
- f. Repeat for second animal
- 4. Texture Discrimination (Day 5 of maternal behavior)
 - a. Learning/Familiarization Phase (10 minutes)
 - i. Place two identical sandpaper textures (coarse) around the inserts, then place the wrapped insert into the outer holes in the behavior chamber.
 - ii. Press record on the camera and place subject in the behavior chamber and shut the door
 - 1. Note the time on the camera when you shut the door, this will serve as your start time
 - iii. Stop recording after 10 minutes from when dark box door was closed
 - b. Retention Phase (5 minutes)
 - i. Remove subject from chamber and place into the home cage
 - ii. Remove inserts and replace with a NEW identical coarse texture as well as a novel texture (fine grain)
 - c. Testing Phase (10 minutes)
 - i. Press record on the camera and place subject in the behavior chamber and shut the door
 - 1. Note the time on the camera when you shut the door, this will serve as your start time
 - ii. Stop recording after 10 minutes from when dark box door was closed
 - iii. Return subject to home cage
 - iv. Clean behavior chamber and textures
 - 1. Mist textures with MB-10 and allow to air dry
 - 2. Clean x 2 with MB-10 and allow to air dry for 5 minutes

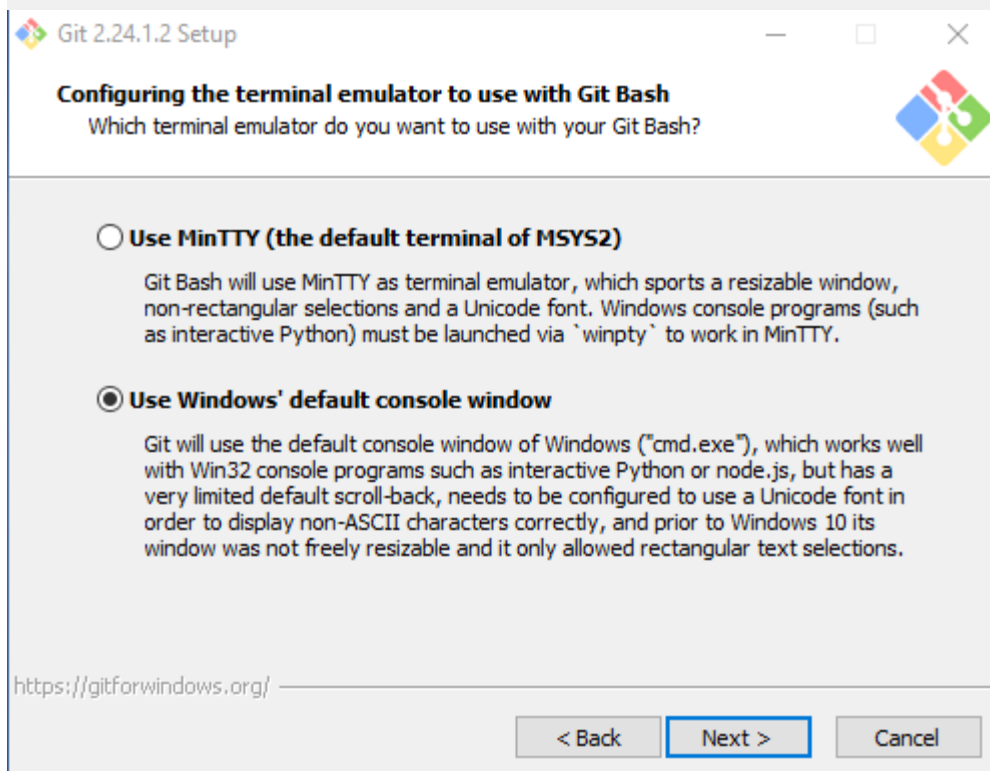
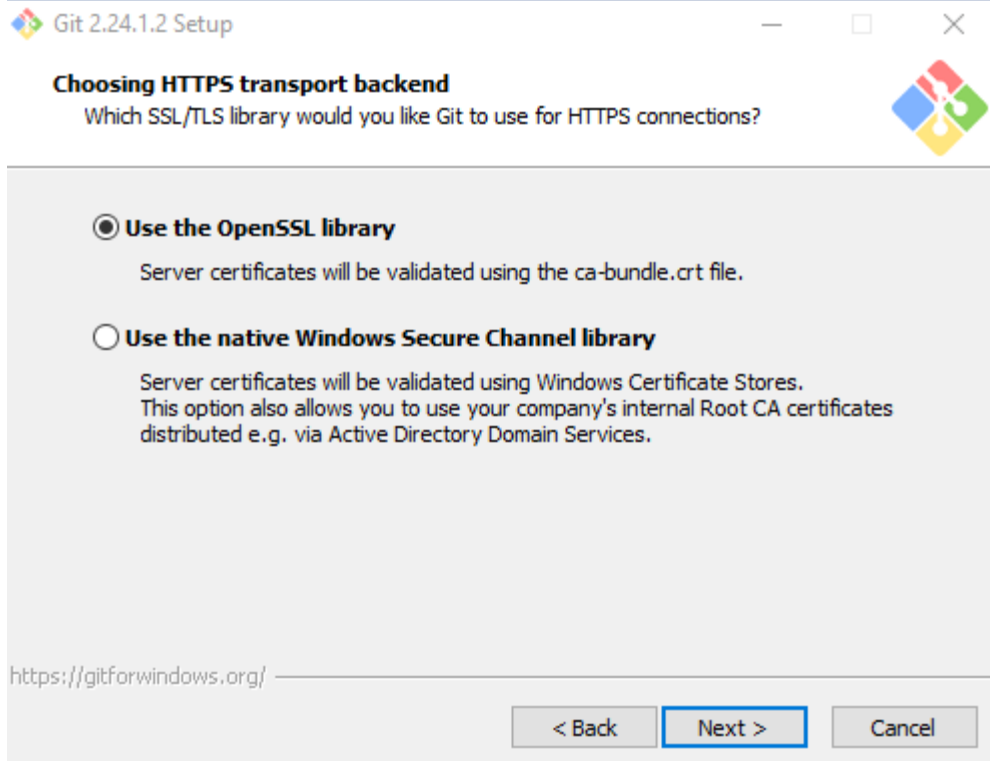
DLC Workflow

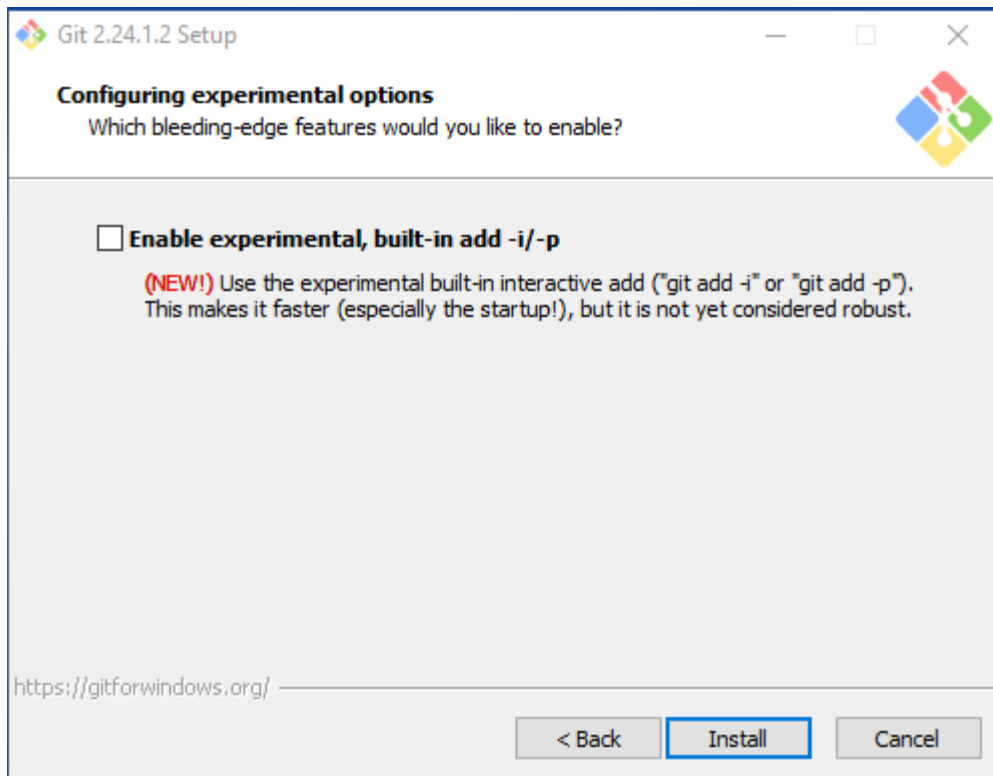
DLC installation

- Install Git bash <https://git-scm.com/downloads>
- Following the setup from here. Make sure you have the options selected as shown in the images below:









- Download anaconda for windows 10 <https://www.anaconda.com/distribution/>
- Install python 3.7.4 (or whatever latest version available for windows 10)
- Once Git is downloaded, open up the Git bash
 - Type the commands:
 - git init (press enter)
 - git clone <https://github.com/AlexEMG/DeepLabCut.git> (press enter)

```
C:\> MINGW64:/c/Users/mmykins

mmykins@Krishnan7050D MINGW64 ~
$ git init
Initialized empty Git repository in C:/Users/mmykins/.git/

mmykins@Krishnan7050D MINGW64 ~ (master)
$ git clone https://github.com/AlexEMG/DeepLabCut.git
```

- NOTE: code is constantly updated and modified by creators for ease of access and installation. If any of these codes are outdated, please refer to GitHub IO

protocol page for the most up to date source:

<https://deeplabcut.github.io/DeepLabCut/docs/installation.html>

- Follow the directions from github page
 - If you have programming experience, then follow the directions at:
 - <https://github.com/AlexEMG/DeepLabCut/blob/master/conda-environments/README.md>

If you do not have experience with programming, please follow these steps:

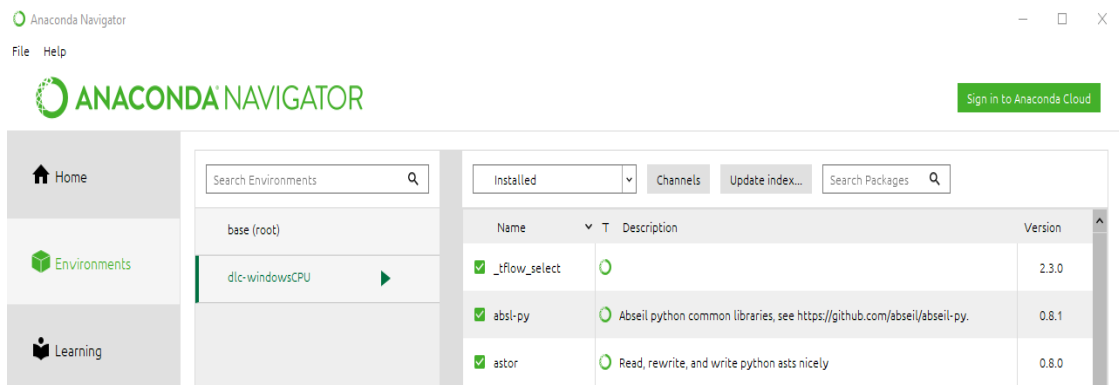
Now, "cd", i.e. go into, the folder named `conda-environments` |

- Cd into the conda-environments where the DeepLabCut file was downloaded.
This sounds straightforward but is not clear to everyone.
- Open Anaconda. In the Anaconda GUI click the arrow on the tab labeled "base" and select open terminal
 - Type cd to find out your current directory
 - In windows explorer find where you downloaded the DeepLabCut to and navigate to the folder named conda-environment
- At the top of the explorer window, you will see the link it should look something like this for examples: C:\Users\mmykins\DeepLabCut\conda-environment **(DO NOT COPY from windows)**
- In the terminal type this command: **** Note your user is specific to you and will not be mmykins****
 - `cd C:/Users/mmykins/DeepLabCut/conda-environment` (press enter)
 - This will move you into the correct directory to upload the dlc-windowsCPU environment. To make sure you are in the right directory type the following command:
 - `pwd` (press enter)
 - your code should print the directory you typed previously.
- Use the appropriate command to upload the dlc environment, depends on if you are running windows

```
conda env create -f dlc-windowsCPU.yaml
```

- type the following command and press enter

- This will import the dlc environment for deeplabcut into the anaconda user interface
- After the environment is done uploading close the terminal. In the Anaconda GUI you



- Type ipython (press enter)
 - import os (press enter)
 - os.chdir ('C:/Users/mmykins/DeepLabCut') (press enter)
 - change the above path to match the directory (folder) where the deeplabcut folder is
- This will cd you into the directory containing the deeplabcut package for the software. Once you have done this you will be ready to import deeplabcut
 - Type the following command:
 - import deeplabcut (press enter)
 - The import will take like a minute to upload.
- Note that if there is an error it's because there was an issue with a package downloading. If this occurs, you will see an error "No module called ' 'exist. You will need to go into the anaconda environment and search for the missing package to download it.
- Once it is finish you can launch the software using this command:
 - deeplabcut.launch_dlc () (press enter)
 - The software will open from there.
 - In the future you won't need to change the directory and will be able to open ipython, import deeplabcut and then launch

Create a training dataset

The following instructions have been adapted and modified from the single animal DLC user guide github.io page:

https://deeplabcut.github.io/DeepLabCut/docs/standardDeepLabCut_UserGuide.html -

**** IMPORTANT NOTE **** Developers constantly update and modify code so please refer to this page for any issues or changes in the process.

1. Open Anaconda as an administrator. To do this, right click on the anaconda icon and click run as administrator. If you search for anaconda in windows and hover over the icon, there will be list of options from which you can select run as administrator
 - a. **** If you do not open as an administrator, you will not have the permission to read and write files using Windows operating system. This will result in the inability to use DLC ****
2. In anaconda, click on environments. Hover over DEEPLABCUT and a green arrow appears. Select green arrow and click open terminal.
3. Type ipython and hit enter
4. Type import os and hit enter
5. Type deeplabcut and hit enter. NOTE: If you do not have a GPU on your computer then an error will appear which says could not find cudart. This is fine and basically says the program could not find a GPU.
6. Type deeplabcut.launch_dlc() and hit enter. The DLC Graphical User Interface (GUI) will pop open. From here on out, everything is done in the GUI.
7. Click 'Create new project' and fill in the name of the project and name of experimenter
 - a. Project names and Name of the experimenter can NOT include special characters, slashes, or dashes (-)
 - b. They can only include letters, and underscores
8. Click Load videos and navigate to the folder which contains videos of interest.
9. Under optional attributes, select copy the videos, and select the directory where the project will be created, if desired. Then press 'Ok'

- a. After pressing 'Ok' it could take a few minutes to copy the videos over. Click the cmd terminal box to make sure code says it is copying over the videos
 - b. After it is finished, a dialog box will pop up that says the project has been created successfully. Press 'Ok'. This will automatically load the project into DLC workspace.
10. Select 'edit config' and the config file will open.
 - a. Here you will define the body parts of interest to track
 - i. Bodypart names themselves can only include letters. DO NOT include space, underscores, dashes, special characters etc.
 - ii. Each Body parts should be defined as shown in the default. For example, here is a list of bodyparts used for tracking mice.
 - Nose
 - Leftear
 - Rightear
 - Shoulder
 - Spine
 - Spine2
 - Lefthind
 - Righthind
 - Tailbase
 - b. Set the pcutoff for your model: a good model should have a pcutoff of 0.8 and above.
 - c. Optional: change skeleton_color: and colormap: to desired color and palette respectively. I personally use blue and plasma, respectively.
 - d. Scroll down to the line 'numframes2pick:' change the number to match the number frames you want to extract from each videos for labeling. Click File -> save and close.
11. At the top of the GUI, click 'Extract Frames'. This will take you to a new tab. DLC will automatically extract frames from your videos via Kmeans using the default

settings. Press 'Ok' and the code will extract the number of frames set in the config file from Step 10d.

- a. Check the cmd terminal to know when all the frames have been extracted
- b. There is also a manual frame extraction where you can toggle through each video and grab whatever frames of interest. Change the 'Choose the extraction method' to manual for this option.

12. At the top of the GUI, click label frames. Click the 'Label Frames' box located above the 'Check Labels!' box.

- a. A dialog box will open asking the load a folder of frames. Select the folder of interest.
- b. Right click on the mouse to place the labels on their respective features of interest. Once you place one label, it will automatically move down the list in sequential order from the list of bodyparts in the config file.
 - i. NOTE: create your list in order that makes sense for ease of labeling
 - ii. If the animal is too far away in the video, then use the zoom function to zoom into the frame. This is advised in general for consistency in labeling.
 - iii. If the animal is not visible, then do not place any labels. Only place labels for each features which is clearly visible.
 - iv. Review 'Help' function for keyboard shortcuts.
- c. Once you are finished with that frame, click 'Next' and this will move you to the next frame. Repeat until you finish the set, then click 'Save' and move onto the next. Repeat this process until all frames are labeled.
 - i. NOTE: training is only as good as the labeler, therefore, take time and consideration and be as consistent as possible when labeling frames.
- d. After labeling all the frames, click Check labels to make sure all frames are labeled properly.

- i. You can view the check labels by opening the labeled data folder in the DLC project. This is done outside of the GUI wherever the project is saved on the computer.
 - ii. If labels printed correctly then proceed to next steps
- 13. At the top of the GUI, click 'create training dataset'. The Hit 'Ok'. In the cmd terminal you should get a message which says the training dataset has successfully been created.
- 14. Upload the entire project folder to Google drive.
- 15. Once the upload is complete, open the config file in Google Drive and update the project path to: /content/drive/My Drive/..... wherever the file location is.
 - a. For example, this is what it might look like: /content/drive/My Drive/dlc_fixes/6WO_maternal-Mike-2023-04-24
 - b. This allows for google colab to find the project once you sync your google drive later on.

Google Colab: Training a DLC Model and analysis

The DeepLabCut team provides a ready-made templates for Google Colab training and analysis which can be found here:

https://colab.research.google.com/github/DeepLabCut/DeepLabCut-core/blob/tf2.2alpha/Colab_TrainNetwork_VideoAnalysis_TF2.ipynb

https://colab.research.google.com/github/DeepLabCut/DeepLabCut/blob/master/examples/COLAB/COLAB_YOURDATA_TrainNetwork_VideoAnalysis.ipynb

If you experience an error with my google colab script below, chances are it's an error that was later corrected later by the team:

https://colab.research.google.com/drive/1eyEhEXZt8vLEYJwXQ1_8CujljaQLuJZV?usp=share_link

Follow the instructions within the colab script for training the model and performing analysis.

****NOTE**** If model performance is not where you want it (high test error, drops in tracking, inverted points etc.) then you will need to refine the predictions and retrain. Please follow the instructions in the next section.

Refining a DLC Model

1. Download the entire DLC project back to the Desktop to refine labels and for retrain to optimize the model.
2. In the DLC GUI, load your project back and press 'Ok'.
3. In the top right-hand corner of the GUI, click 'extract outlier frames'. Click 'Choose the videos' and select the videos you want to perform corrections on.
4. Specify the video type for your videos and then select the algorithm for outlier extraction. There are four options.
 - a. Jump- identifies larger jumps than distance to the estimated data (epsilon) in any body part
 - b. Fitting- fits a autoregressive integrated moving model average (ARIMA) to the data and computes the distance to the estimated data (epsilon). Distances larger than epsilon are identified as outliers.
 - c. Uncertain – grabs outliers which do not meet the defined pcutoff values. If $p = .9$, then it will grab select number of frames where the likelihood is below .9.
 - d. Manual- manual grab frames which have incorrect labels. Requires toggling through the video and selecting instances.
5. Jump and Fitting are robust methods for outlier correction. View the Help function for help in deciding which function to use.
6. Once you select a specific algorithm, press 'Ok'.
 - a. If you choose manual, you will be prompted with a new window to extract outliers from the videos.
 - b. If you choose any of the other 3 options, the software will automatically go through the videos and search for outliers based on the criteria.
 - i. NOTE: you can run outlier correction using multiple options, but only one method at a time.
 - c. In the cmd you will be prompted with a question for each video which will say "we found X number of outliers, would you like to proceed with extracting X amount? ". Type Y in the cmd and hit enter. Repeat for all videos as needed.

7. After you finish extracting outliers, at the top of the GUI, click 'refine labels'. Then click 'LAUNCH'. This will open a new window where you can upload the extracted outliers and correct them.
8. Navigate to the labeled-data folder and select the video you want to correct. In the folder there will be a 'machinelabels.h5' file. This is the file type DLC uses to store the potential outlier for correction. Click the file and select 'Open'.
9. A dialog box will open with instructions for outlier correction. Proceed by hitting 'Ok' or hitting enter. Another dialog box will open and ask you to set a confidence threshold. This threshold will help you to see where issues are in tracking.
 - a. Open circles indicate a prediction below threshold
 - b. Full circles indicate a prediction equal or above threshold
10. Left click and drag to move and correct predicted labels if needed.
 - a. If you are having trouble locating a label, increase the marker size by using the slider in the top right-hand corner of the GUI.
 - b. If a label is predicted, but the feature of the mouse is not visible, you can delete it by clicking on the middle cursor of the computer mouse. A dialog box will appear which warns that once a label is deleted, you cannot recover. Confirm you are deleting the right label and press 'Yes'.
11. Repeat step 10 for all of the frames and then click 'save'. You will be prompted with a dialog box that says do you want to correct another file. Select yes to continue with correction.
12. Once you have corrected all of the extracted outliers, click the 'MERGE' datasets button. This will merge the corrections with your labeled data. At the top of the GUI click 'label frames' and then select 'check labels'. Make sure the labels are plotted properly. Click the create training dataset tab and then press 'Ok'.
13. Upload the entire DLC project back to google drive and replace it with the old instance when prompted. Repeat Google Colab training and analysis and outlier correction until desired test error is achieved.

Datavyu behavioral quantification

All Datavyu behavioral quantification was performed in Datavyu version v1.3.7. Datavyu can be downloaded from here <https://github.com/databrary/datavyu/releases>. For consistency in labeling whisker interactions with textures/objects, we drew a 3 cm radius around the left and right texture/object. Literature indicates the average mouse whisker length is approximately 3 cm and is used to define whisker interaction. We then analyze the videos in datavyu to quantify interactions with objects/textures.

1. To create the file, go to file -> new and name the file after the video being analyzed. This is called an opf files and is how datavyu stores time stamped interactions.
 - a. To remove observer bias, we blinded observers to experimental conditions by randomly assigning a number to the video.
2. In the data view controller window click -> add data and navigate to file location of the video you want to analyze.
3. In the main datavyu window, click the plus and add a new column called left (these will represent interaction with the left object/ texture in the video), then add another new column called right (these will represent interaction with the right object/texture in the video).
4. Set the start time in the left and right column as when you hear the door click in the video. Score interactions for the next 10 minutes from the start time.
 - a. For example: If the door closes at 00:09:10, the end time should be 10:09:10 or as close as the video will allow.
 - b. At 10:09:10 you will add an interaction on the left and right column called 'end'
5. Navigate throughout the video and score each individual interaction. To set an interaction follow these instructions:
 - a. Click onset for when the interaction starts and then click offset for when the interaction stops, or transitions to a new type of interaction such as whisking to forepaw, or forepaw to whole-body climbing.

- i. Once you are comfortable with the data view controller, you can turn on number lock for easy scoring using the number keys shown here <https://datavyu.org/user-guide/guide/controller.html>
 - b. Make sure you are labeling the left interactions in the left column and right interactions in the right column. This is an important distinction later on as the right texture/object is replaced with the novel texture in the testing phase.
- 6. Interactions are scored based on the following criteria.
 - a. 'w' for whisker- Whisker Interactions are defined as when the mouse is within 3 cm of the texture/object. Once the mouse is within the 3 cm box the interaction is labeled as 'w' within datavyu.
 - b. 'b' for paw- As soon as the mouse puts a paw on the object/texture, set offset for 'w' and set onset for 'b' even if this is one big interaction, these distinctions are important.
 - c. 'c' for climb – when the forepaws and hind paws of the mouse have lifted the ground and are in contact with the object/texture. All 'b' and 'c' should be surrounded directly by 'w' unless the mouse jumps from farther out to climb because they use their whiskers before they use their paws, and they usually use their paws before they climb. They also use their whiskers after paws and/or climbing.
 - d. 'w-bite' – physical biting of the textures

General workflow for Datavyu analysis:

1. make a new folder for each video + your opf file with same name as video (DO NOT export to excel yet)
2. move folder into 'need to be checked' after you're done so another observer can check your scoring.
3. for checking other's work, check the ones in their 'need to be checked'
4. save corrections with exact same file name + your initials

5. export to excel file (open excel file to make sure there are data!)
 - a. when exporting the data from datavyu, name the file by the code only, no initials and use lowercase letters only. For example, a file named "C_AM" would be called "c". When exporting by frame, the file would be named "c_frame".
6. then move checked folder into 'done' folder.
7. After the file is checked it is decoded in R for analysis.

Code repository

All codes utilized throughout the dissertation are stored on the Krishnan Lab Server.

Open access to the code will be available on Zenodo through a reserved DOI which will be included with each Chapters publication:

Surgery manipulation

All surgery experiments were performed by Dr. Billy Lau. The following protocols are notes and instructions that I wrote while observing him perform the experiments.

1. Put the mouse in a new cage and give it a new card to distinguish it for surgery
2. Going into surgery room, turn on computer (no login), open Angle Two (software for setting stereotaxic coordinates)
3. Turn on the lights for the microscope; turn on heater (blue box). Make sure the sensor from the heater is touching the heating pad which is under the platform on the microscope
4. Fill up the iso container
 - a. You want to have at least one full circle of iso when you start ~70 mL
 - b. 1 animal is about $\frac{1}{2}$ circle ~ 35 mL
5. Turn on the air pressure machine and turn on the air (orange knob)
 - a. If the pressure is too high, then tube will pop off. You can use plumbers tape to help seal it back up.
 - b. You want the pressure to measure ~ 1 on the air pressure machine
6. Place kem wipe down where the animal will lay
7. Open the autoclave bag but do not touch anything inside
8. Make sure you have autoclaved bone wax
9. Adjust the iso settings per genotype, refer to blue note on iso tank for reference
10. If the mouse is breathing too fast, adjust iso by one click
11. Iso and O₂ are pushed to the mouse and the vacuum pulls the iso and O₂ away from the mouse and you. The vac value should be set to 2
12. First need to Knockout the mouse with .5 mL of iso, and .5 mL of iso in the tube
 - a. When the mouse goes into the chamber take her ASAP once she goes down
13. Begin shaving the skull, after shaving use tape to remove remaining hair
 - a. If the mouse wakes up during this process, put her back under iso for 5 seconds and proceed
 - b. Be careful not to damage the eyes while shaving the skull

14. After shaving, place the mouse on the platform and secure her on the mouthpiece by placing the top teeth into the groove on the mouthpiece. Get the mouth locked in and turn on O₂, iso and the vacuum, in that order
15. Push the mouse head into the chamber but don't push in all the way because this will place pressure on the nose and dislodge the teeth from the clamp
16. Lock earpieces around the jaw, but don't do it too tight because this can restrict breathing, if you notice the mouse isn't breathing then loosen the pieces
 - a. Hets will have more shallow breathing and WT will not be as shallow, adjust iso accordingly if needed. You want about ~ 1 beat/s
17. Put eye lubricant on the eyes so that they don't dry out during the procedure
18. Wash the incision site with beta-iodine soaked cotton swab then use 70% ethanol
 - a. Rotate the cotton swab so that you're not using the same side each time
19. Remove the first layer of wax paper (cause its dirty), and drape the animal with wax paper
20. Change your gloves to sterile gloves. To place the sterile gloves on fold the glove out only touching the base, and using one hand, slide the hand into the glove. Place the second glove on with the assistance of your gloved hand
 - a. If your hands are sweaty, use the baby powder to help you in this process

From this point on everything has to be *****ASEPTIC*****. At this point, everything you touch should be sterile. If you touch something not sterile then change your gloves

21. Everything you touch will be from the packet. Place autoclaved foil around all parts of the microscope, iso tank, and surfaces that you will use/touch
 - a. Large pieces are for the knobs
22. Make a window in the draping to get access to the brain
23. Place ear clips on the ears to create tension on the skin. Lift up skin and make the first incision down the midline of the skull
24. Adjust microscope to find bregma (blood vessels that make triangle at the rostral part of the brain) and lamda

25. Place the needle from the top right of the box into the electrode to use as a landmark to find bregma and lambda. Insert the needle by pushing it all the way up
 - a. may need to loosen or tighten accordingly
26. Lower needle to as close to bregma and set the coordinate in angle two. Move the electrode down to find lambda
 - a. These are the injection coordinates for S1BF
 - i. Map number 32 (-0.11 mm B, 3.25 mm L, .55 mm D)
 - ii. Map number 42 (-1.31 mm B, 2.75 mm L, .55 mm D)
27. Pull the skin back from the skull gently, making sure not to shift the position of the skull
 - a. If the skull position shifts, then you need to reset bregma and lambda
28. You can insert cotton tips in between the skin and the skull to hold the skin away from the injection sites.
29. Mark each injection site with a sharpie, this is where you will use the drill to shave down the skull.
 - a. Mark all sites prior to starting to drill.
30. Load 1 uL of reagent (penicillinase or ChABC) onto the top of the electrode and let it fall in while you drill, flick gently at the top if it is stuck.
 - a. Do not flick too hard or it will crack the electrode
31. Place drill bit in place all the way in for best control. Wrap the drill in foil, turn on the drill, and use the pedal to control the speed. Use both hands to steady the drill.
32. Zoom into marked injection site, orient the microscope to the skull and shave the skull until you see blood vessels. Start to remove the remaining skull with the fragile forceps.
 - a. Use the stage to steady your hand while doing this.
 - b. Pull broken bone away from the brain and be careful not to stab forceps into the brain
33. Prepare the electrodes for injection. To do this pump air into the electrode to move the solution down to the tip

- a. Before injection, make sure solution is coming out of the electrode, use a test pump to see if the solution is coming out. Once it is out use a sterile cotton tip to soak up the solution at the tip.
34. Lower the electrode to the brain surface and test for resistance to ensure all skulls has been removed.
 - a. If there is no resistance, then proceed by lowering the electrode into the brain.
 - b. If there is resistance, then there is still skull fragments on top of the surface of the brain. Remove these pieces and repeat.
35. Once the electrode is lowered into the brain, do 10 injections, approximately 1 s apart (15 ms @10 puffs is equal to 1 puff per second)
 - a. Sometimes if you do an airpuff, it might bring the solution back up if there is too much pressure.
 - b. If this happens do 2 consecutive puffs and pause
 - c. You can also try gently moving the electrode up and down
 - d. If nothing is coming out from the electrode, increase pressure in 10 ms intervals up until 60 ms, to see if the solution is coming out
 - e. Increase pressure to 200 ms. If no solution is coming out then gently break the tip of the electrode using the fragile forceps, and test again.
 - i. If the solution is coming out, then reduce back down to 15 ms
 - f. If the first puff has more volume than intended, then you need to adjust the number of puffs to equal the same desired volume
36. Once the 10 puffs are finished, leave the electrode in place for 3-5 minutes and then slowly remove the electrode from the brain
37. Repeat steps 35-36 for the other side of the brain, depending on experimental conditions
38. Break a cotton swab in half and use it to apply a small amount of bone wax to the injection site.
39. Next take out the suture. Cut the tip of the suture as this was exposed to air and not sterile.

- a. Make the first puncture with the suture behind the right eye and cross over to the skin behind the left eye. Thread the needle.
 - b. Repeat a., doing another suture to bring the flaps of skin together.
- 40. Use forceps to guide the skin glue between the two suture sites. This will help the skin adhere together. Inject of saline solution with a 31-gauge needle into the mouses back
 - a. Pull back the skin of the mouses back, avoiding the spinal cord, and slowly inject
 - b. 1 uL per gram, for WT it is approximately 20 uL
 - c. This is done to rehydrate the mouse
- 41. Inject carprofen using .3 mL syringe via IP into the back hindlimb of the mouse
 - a. 10 uL per animal for Het and 20 uL per WT
- 42. Place the animal in a single housing cage with a heating pad underneath the cage.
- 43. Repeat for next animal

VITA

Michael Jason Mykins was born and raised in Norfolk, VA. He graduated from Grassfield High School Governors STEM academy in 2013 and was the first person in his family to graduate High School and go to college. Michael moved to Blacksburg, VA to attend Virginia Tech as a Virginia Tech Presidential Scholar where he focused his initial studies in biophysics and later, cellular neuroscience. In 2015, he began undergraduate research under Dr. Gregorio Valdez, at the Fralin Biomedical Research Institute in Roanoke, VA. In Dr. Gregorio Valdez's lab, he worked on a collaborative project investigating the effect of a small molecule on motor retention in a mouse model for Amyotrophic Lateral Sclerosis and an independent project investigating the role of Fibroblast Growth Factor Binding Protein 1 (FGFBP1) on excitatory and inhibitory synapses in the developing mouse hippocampus. After graduating in 2017 with his Bachelor of Science in Neuroscience, he accepted a research technician position in the lab of Dr. Blaise Costa at Edward Via College of Osteopathic Medicine in Blacksburg, VA. While in this position he published a co-author paper on the therapeutic value of cranial osteopathic manipulation for clearing amyloid beta in an aged rat model for Alzheimer disease. Michael pursued his education further by joining the Biochemistry & Cellular and Molecular Biology department at the University of Tennessee Knoxville in 2018. There, he joined the lab of Dr. Keerthi Krishnan, where he investigated age dependent regression in a female mouse model for Rett syndrome. During his tenure, Michael received multiple awards to support his research and travel to international conferences to present his work. Michael served as secretary for the BCMB GSO and heavily participated in community outreach through local elementary schools and skype a scientist. Despite a pandemic, and an unexpected diagnosis of Type 1 diabetes, Michael maintained productivity and determination and published 1 coauthor and two first author manuscripts, and two first author preprints. Michael has accepted a post-doctoral fellowship with Dr. Ted Dawson and Dr. Valina Dawson at the Johns Hopkins School of Medicine in Baltimore, MD. Michael will return to his previous research background in neurodegenerative disorders but will use the skills from his dissertation training to advance new directions in Parkinson's research.