Developing whisker movement protocols for the study of rodent models of neurological disease

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Abstract

Laboratory rodents are a valuable tool for research in biomedical science and are key to improving our understanding of neurological disease. Behavioural studies are fundamental for revealing the connectivity between molecular and genetic changes and the system-level response of affected individuals. However, behavioural studies in many rodent models reveal quite variable outcomes. One way to better capture sensorimotor and executive deficits that are common in rodent models of neurodegenerative disease is by using a highly quantitative, repeatable behavioural task. I propose that measuring whisker movements in rodent models offers an easy, quick and robust way to capture elements of motor, sensory and cognitive disturbances. In this thesis, I will make recommendations for the application of whisker movement measures for the study of rodent models of neurological disease, especially developing methods to standardise and automate the method. I will also discuss the integration of whisker movements with other behavioural tasks and within the context of general exploratory behaviour. My thesis will consist of three experimental chapters, using three rodent models, including: 3xTg-AD mice (a model of Alzheimer's disease), MIA rats (a model of neurodevelopmental disorders) and reeler mice (a model of disrupted development of cortical layer formation). In the first experimental chapter I present the whisker tracking protocol and increase its automation by removing manual scoring. In the second experimental chapter I, for the first time, demonstrate treatment differences using the protocol with rats, and integrate it with a sequential object task. In the third experimental chapter, I integrate the whisker movement protocol with a habituation task and place whisker movements within the general locomotor-exploratory measures. Overall, I documented whisker movement deficits in all the rodent models tested. The findings in this thesis suggest that measuring whisker movements is a powerful behavioural measurement tool, capable of revealing age-related and treatment effects, as well as sex and object differences. However, measuring whiskers in the standard task might not suit all rodent models, and further exploring how whisker movement measures might combine with other tests could be useful, especially with the novel object recognition and social tasks that are thought to be translatable to humans.

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List of abbreviations

3Rs – three Rs

5-HT6 – 5-hydroxytryptamine receptor subtype 6

AD – Alzheimer's disease

ADHD – attention deficit hyperactivity disorder

ANOVA – analysis of variance

ART – automated rodent tracker

CC – corpus callosum

CIA – contact-induced asymmetry

DAPI - 4′,6-diamidino-2-phenylindole

DC – during-contact

DF – degrees of freedom

GABA – γ-Aminobutyric acid

HAB1 – first habituation

HAB5 – fifth habituation

HTA – head turning asymmetry

IR – infrared

IRt – intermediate band of the reticular formation

LMEM – linear mixed effect model

M1 – motor cortex

MIA – maternal immune activation

NaCL – sodium chloride

NDD – neurodevelopmental disorders

NOE – novel object exploration

NOR – novel object recognition

OF – open field

PC – pre-contact

PC-DC – contact-related changes

PCP - phencyclidine

Poly I:C – polyinosinic:polycytidylic acid

RA – rapidly-adapting

RRC – rete-ridge collar

RS – ring sinus

RW – ringwulst

S1BF – primary somatosensory cortex barrel field

SA – slowly-adapting

SA1 – slowly-adapting type 1

SA2 – slowly-adapting type 2

SEM – standard error of the mean

SOM – somatostatin

TB – TRIS buffer solution

TG – transgenic

TRIS – tris(hydroxymethyl)aminomethane

VGAT - vesicular GABA transporter

VGLUT1 - vesicular glutamate transporter 1

VIP – vasoactive intestinal peptide

vIRt – vibrissae zone of the reticular formation

VP – vasopressin

WT – wildtype

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 Thesis introduction

Studies on laboratory mice (*Mus musculus*) have contributed significantly to the understanding of human biology and health (Fox et al., 2006; Morse, 2007; Perlman, 2016), and rodent models are a powerful tool for research in biomedical science. This is mainly due to their genetic similarities to humans (Mouse Genome Sequencing Consortium, 2002), the ability to create transgenic, knock-out, and knock-in varieties, as well as the ease and relatively low expense of keeping and breeding them (Burns et al., 2015). Rodent models are key to reducing complexity in the study of age-related progressive neurodegenerative disorders such as Alzheimer's Disease (AD), the most frequent form of dementia in elderly people (Fiest et al., 2016). They help to enhance our understanding of neural and behavioural changes during disease progression, and to develop novel therapeutic targets (Scearce-Levie et al., 2020).

Behavioural studies are fundamental to revealing the connectivity between molecular and genetic changes and the system-level response and capabilities of affected individuals. However, behavioural studies in many rodent models reveal quite variable outcomes, especially in terms of motor and cognitive tasks involving spatial learning and memory (Fertan et al., 2019c; Stover et al., 2015). Therefore, a better understanding of the behavioural manifestations of, for example, neurodegenerative disease, that occur in those rodent models is needed.

One way to better capture and describe motor, sensory and cognitive deficits that are common in rodent models of neurodegenerative disease is by using a highly quantitative, repeatable task. Measuring whisker movements in mouse models has been suggested as an easy, quick and robust way to capture elements of motor, sensory and cognitive declines (Simanaviciute et al., 2020). It has previously been demonstrated in mouse models of Amyotrophic Lateral Sclerosis (Grant et al., 2014), Huntington's Disease (Garland et al., 2018), anxiety (Grant et al., 2016), Alzheimer's Disease (5xFAD and 3xTg-AD, Grant et al., 2018b; Simanaviciute et al., 2020), as well as Cerebellar Ataxia, Somatosensory Cortex Development disorders and Ischemic stroke (all tested by myself during my placement year, and published in Simanaviciute et al., 2020).

Laboratory mice and rats rely on whiskers as their primary sense of touch (Grant and Arkley, 2016). As well as sensing, whiskers are actively controlled; they can move rhythmically to-and-fro in a process called whisking, which occurs at up to 25 Hz in mice and is amongst the fastest movements that mammals can make (Mitchinson et al., 2011). In many disease models, this whisking motion can be disrupted and might be indicative of motor deficits (Grant et al., 2014; Garland et al., 2018; Simanaviciute et al., 2020). Mice also precisely control their whiskers during object exploration (Carvell and Simons, 1990; Mitchinson et al., 2007; Grant et al., 2009), so as well as making simple, cyclic movements, they can alter the timing, spacing and positioning of their whiskers to maximise sensory information (Carvell and Simons, 1990; Grant et al., 2009; Mitchinson et al., 2007; 2011). Therefore, as well as a model of sensory processing, whiskers are also a good system from which to study motor control and exploratory behaviours. When a mouse contacts an object with their whiskers, they tend to decrease whisker angles and whisker spread, increase whisker asymmetry and amplitude, and slow whisker speeds (Mitchinson et al., 2007; Grant et al., 2009; Grant et al., 2018a) – these changes allow many whiskers to gently contact a surface for longer, hence increasing the quality of the sensory information captured (Grant et al., 2009). The positioning and focussing of many whiskers on to an object is thought to be associated with attention (Arkley et al., 2014; Mitchinson and Prescott, 2013). Therefore, whisker movements and positioning on objects may reveal sensory and cognitive deficits in mouse models (Simanaviciute et al., 2020).

I propose that measuring whisker-related exploratory movements offers a useful way to characterise highly quantitative behavioural changes in mice. Not only does it measure an intrinsically motivated exploratory behaviour, which should enable universal measures despite the disease symptoms, but it also takes only a short amount of time to capture results. It is a relatively automated process, which should also reduce subjectivity and variation. Within my thesis, I will make recommendations for the application of whisker movement measures for the study of rodent models of neurological disease, in particular focussing on:

i. standardising the protocol,
ii. automating the protocol,
iii. integrating whisker movements with other behavioural tasks and placing them within the context of general exploratory behaviour.

As well as a literature review, my thesis will contain three experimental chapters, reporting novel whisker movement measures in three rodent models:

- Chapter 3: 3xTg-AD mice – a mouse model of Alzheimer's Disease,
- Chapter 4: MIA rats – a Maternal Immune Activation rat model of neurodevelopmental disorders, including autism, schizophrenia and ADHD,
- Chapter 5: Reeler mice – a mouse model of neurodevelopmental disruption.

These rodent models reveal particularly challenging behavioural phenotypes, and results from previous behavioural tests have not been robustly repeated across studies. Therefore, they are useful models for me to investigate with my whisker measurement techniques.

The final chapter of my thesis will be a discussion, where I bring together my findings and observations into a series of recommendations for the future study of whisker measurements in the field of neurological disorders.

1.2 Whiskers – not just simple hairs

Whiskers differ from pelage hairs (or fur) in several ways: they are longer, often located in the facial region, and their follicles are 5-6 times larger and highly innervated (Ahl, 1986; Andres and von During, 1973). But perhaps more importantly, whiskers are different from common hair or fur because they can ***sense*** and ***move***. Nearly all mammals have whiskers at some point of their development (Cave, 1969; Ahl, 1986). Even in humans, striated muscles attached to hair follicles are found in the upper lip, suggesting that we might have once had the ability to voluntarily control those muscles (Tamatsu et al., 2007), just like many whiskered animals. However, the main touch receptors - Merkel cells - are not found around the upper lip in humans, but rather on the fingertips. Therefore, to better relate to the experience of whiskered animals, we can compare whisking to human touch-sensing of the fingertips.

When we use our fingertips, we employ different strategies such as stroking, palpating or scanning in space, to find and identify an object and understand its size and texture. This is very similar to what rodents do with their whiskers. Indeed, whiskers are essential to ***survival*** in nocturnal rodents, especially as they cannot rely on their vision in the dark. For instance, we have known for a very long time that rats have great difficulty learning a maze without their whiskers, more so than when deprived of other sensations (Vincent, 1912). Similarly, Schiffman et al.

(1970) found that rats' tactile sensations prevail over vision when their inputs contain conflicting information in a depth perception task. Furthermore, laboratory rats are born with whiskers (but with closed eyes and ears; Park, 1970) which constantly grow and regrow if plucked. The speed of this growth does not reduce until the near-death life stages, suggesting the importance of vibrissae throughout the life cycle of an animal (Ibrahim and Wright, 1975; Ahl, 1986).

In addition, in laboratory mice, whiskers must be intact until at least the fourth post-natal day for normal somatosensory cortex development (Woolsey and Wann, 1976; Weller and Johnson, 1975; Ahl, 1986). Whiskers seem to be involved in aggressive behaviours and fighting in laboratory mice, where only whiskered animals fight, and losers get punished by the winners barbering their whiskers (Ahl, 1986). Moreover, a lack of whiskers interferes with a rat's ability to keep the nose above water when swimming (Ahl, 1982) and can impact the results of several standard behavioural tests in a mouse strain commonly used as control animals for various studies (C57BL/6, Haridas et al., 2018). Rauschecker et al. (1992) reported that mice deprived of vision since birth relied on their whiskers even more than healthy ones, which resulted in their whiskers being longer and thicker than in the control mice.

These are only some examples from a lengthy list supporting the idea that whiskers are the most important sensory organ in laboratory rodent species.

Whiskers can be classified as primary, i.e., attached to the mystacial pad (upper lip), or secondary, attached to other places of the face or body (Pocock, 1914). The focus of this thesis is only on the primary whiskers because they are the only movable whiskers used for active sensing. In this region, there are two further types of whiskers: micro-vibrissae, the shorter whiskers in front of the mouth, and macro-vibrissae, the long whiskers structured in rows and columns (Brecht et al., 1997). Primary macro-vibrissae are of interest to me because of their orderly fashion and each whisker's connection to a single muscle, allowing for precise and active movement to collect information about the environment (Berg and Kleinfeld, 2003).

1.3 A model sensory system

Neurons from the whisker follicles are the most represented structure in the trigeminal ganglia (Kruger and Michel, 1962; Nord, 1967; Ahl, 1986), once again

emphasizing this system as the primary sensation in rodents. This is due to each follicle being innervated by between 150 and 200 myelinated axons (Vincent, 1913; Lee and Woolsey, 1975; Dörlf, 1985; Rice et al., 1986; Lichtenstein et al., 1990). Perhaps the most striking feature of whiskers is that they are represented at every neuroanatomical level. Neurons project from the mechanoreceptors in the whisker follicle through the trigeminal nerve into the brain stem, the thalamus, and finally into the somatosensory cortex contralateral to that whisker's side (Jeanmonod et al., 1977; Ahl, 1986). Every macro-vibrissa is represented by a unique barrelette in the brain stem, a barreloid in the thalamus and a barrel in the cortex, keeping the structural arrangement of rows and columns, resulting in a precise mapping from the mystacial pad throughout the hierarchy of the brain (Figure 1-1; Figure 1-2; Weller and Johnson, 1975; Jeanmonod et al., 1977; Ahl, 1986). Some ipsilateral connections exist too, suggesting that information from both mystacial pads (of the left and the right sides of the snout) can be compared (Pidoux and Verley, 1979; Ahl, 1986).

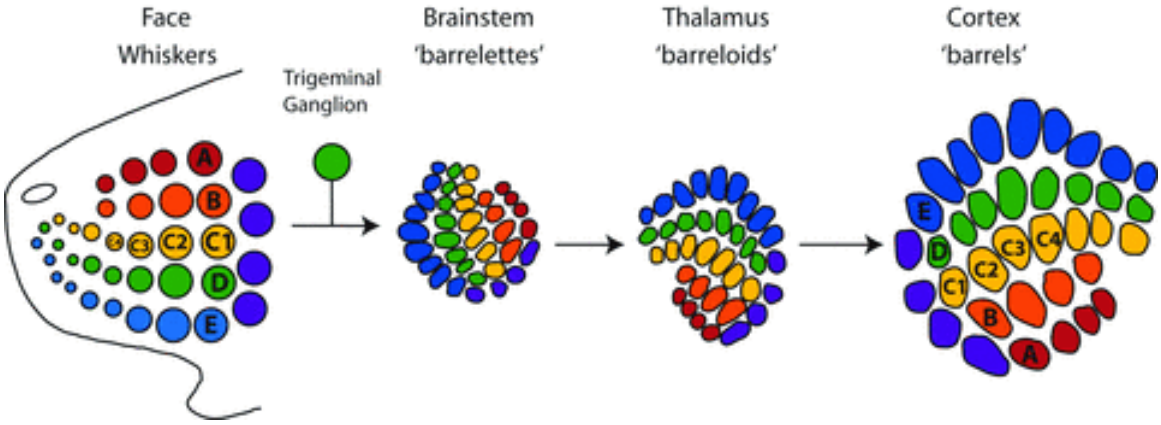


Figure 1-1 **Organisation of whisker representation in the brain.** The topographical map made of rows and columns is preserved from the original arrangement in the mystacial pad to the barrelettes in the brainstem, barreloids in thalamus and barrels in the somatosensory cortex. Adapted from Li and Crair (2011).

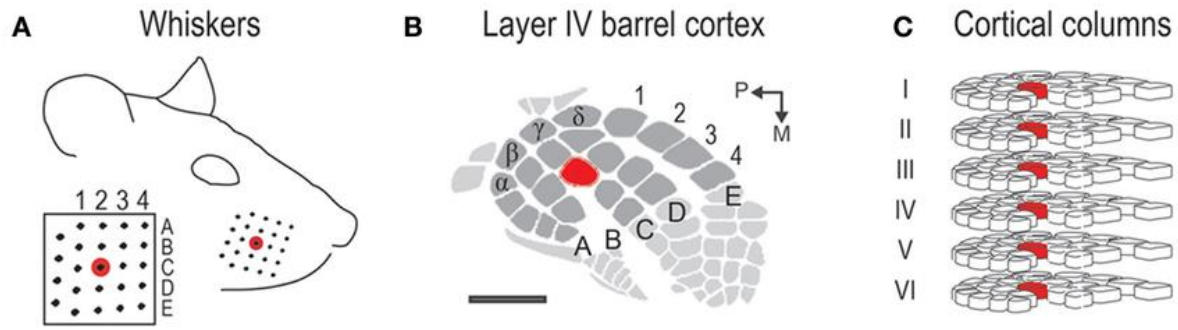


Figure-1-2 **Barrels represented throughout the cortical layers.** This figure illustrates one C2 whisker represented at the mystacial pad, in layer IV of the barrel cortex and through the cortical columns. Adapted from Chen-Bee et al. (2012).

There are some differences in the properties of whisker representations at different brain levels. Barreloids in the thalamus are more selective to angles than barrels in the cortex, while trigeminal ganglion cells respond more selectively to whisker movement deflections and angular direction, and thalamic and cortical cells respond to more deflection angles than primary afferent fibre (Lichtenstein et al., 1990; Adibi, 2019 for a review). These distinctive central and peripheral response properties allow for progressive integration from different receptors and whiskers (Lichtenstein et al., 1990). While at the periphery each sensory neuron is innervating only one whisker follicle, the integration of inputs from more than one neuron and more than one whisker is taking place centrally (Lichtenstein et al., 1990; Brecht, 2007). Convergence of inputs and cross-whisker inhibition - the process of the nearby whiskers supressing the discharges of a targeted whisker as a mechanism of edge detection - starts in the thalamus and even more of that inhibition occurs at the cortex level from local inhibitory neurons (Lichtenstein et al., 1990; Lavallée and Deschênes, 2004).

Early studies were interested in what the first order neurons in the trigeminal system encode. Zucker and Welker (1969) suggested peripheral location, deflection direction, onset and termination, amplitude, speed, duration, repetition rate and temporal pattern of the mechanical stimuli, as well as the movements of the whiskers, are all encoded in those neurons. This classic study also identified ‘five categories of vibrissae units’ in the trigeminal ganglia which respond to the stimulation of one whisker only. Neurons that are the most selective for whisker direction are, in general, the most responsive ones in terms of more spikes per stimulus from single-whisker stimulation compared to cells that are not as directionally sensitive (Lichtenstein et al., 1990). Several types of

mechanoreceptors have been identified in the whisker system and include both slowly (SA) and rapidly adapting (RA) types. In the trigeminal nerve, ~75% are SA cells which are directionally tuned, while the other ~25% are RA mechanoreceptors (Lichtenstein et al., 1990). In the thalamus, there is only 37% of SAs (Simons and Carvell, 1989) due to the convergence of information.

A more recent study from Soneketsu and Gu (2020) used a single-fibre recording technique, which detects responses from a single mechanoreceptor. They have identified two types of slowly-adapting mechanoreceptors SA1 and SA2, as well as rapidly-adapting receptors. RAs were found to only activate during the dynamic phase, i.e., when the stimulus is initiated, hence they are suitable for detecting short mechanical stimulation. SA1 and SA2 mechanoreceptors fire during both dynamic and static phases of whisker deflection; SA1s, however, fire irregularly, while SA2s respond regularly. SA mechanoreceptors can detect graded mechanical stimulus or encode stimulus intensity and length, as they keep firing throughout the stimulus application. All mechanoreceptors in the whisker follicles are frequency independent and encode inputs from 10 to 100 Hz (frequency range during rodent environmental exploration) equally well. Receptive fields for RA and SA1 firing are mainly located in front of the ringwulst (Figure 1-3), while SA2 receptive fields are mainly in the rear of the ringwulst.

Slowly-adapting and rapidly-adapting is just one way of classifying these mechanoreceptors according to their function; however, several different cell types have been identified that correspond to these classes. For instance, Merkel discs have been found in front of the ringwulst and rete-ridge collar (skin surface epidermis at the mouth of the follicle opening) of whisker follicles (Ebara et al., 2002, Furuta et al., 2020), as well as in human epidermis (Zimmerman et al., 2014). They have Piezo2 ion channels, which transduce mechanical force into Ca^{2+}-action potentials (Woo et al., 2014). Merkel discs then send the information down via serotonergic synapses through the SA1 afferent endings (Ikeda et al., 2014). Additionally, SA1 endings have inotropic and metabotropic serotonin receptors that are highly sensitive and work in synergy to encode tactile stimuli at the frequencies of active whisking; information from both sources is then relayed into the trigeminal ganglion (Chang et al., 2016; Sonekatsu and Gu, 2020). The location of Merkel discs at the whisker follicle is one of the main factors of their response properties: ring sinus, or RS-Merkel endings, are the only slow-adapting mechanoreceptors and respond the fastest and with the largest magnitude, while

rete-ridge collar, or RRC-Merkel discs show longest latency and lowest magnitude of all mechanoreceptor responses and are rapidly-adapting (Figure 1-3; Furuta et al., 2020). This is likely due to the surrounding tissue properties and not the differences between Merkel discs (Futura et al., 2020). The other two types of mechanoreceptors found at the whisker pad are lanceolate and club-like endings which are intermediate in their response magnitude and delay (Furuta et al., 2020).

The location is not the only important element in response property differences: mechanoreceptor internal properties, geometry and orientation in the follicle as well as their position relative to other mechanoreceptors and surrounding tissue must be considered. For instance, RS-Merkel and lanceolate endings are closely located in the whisker follicle (Figure 1-3) and likely receive similar mechanical inputs, yet they have differing response properties. RS-Merkel receptors respond to direction, and they are tuned to angles, meaning they have a preferred angle to which they respond the most. Moreover, they can respond in an ON or OFF manner and these responses are variable and do not exhibit a correlation between latency and magnitude (Futura et al., 2020). Merkel receptors are ideally positioned to respond with particular characteristics to the bending moment as that is a directional force. Meanwhile, the preferred directions of the lanceolate and club-like endings are not correlated to their locations in the whisker follicle, as they respond to the axial force of which magnitude is direction independent (Furuta et al., 2020). All mechanoreceptors were found to have some tuning, although of different sensitivity to specific stimuli (Futura et al., 2020). Overall, this sensory system allows for a wide variety of responses, depending on the types of receptors and their properties.

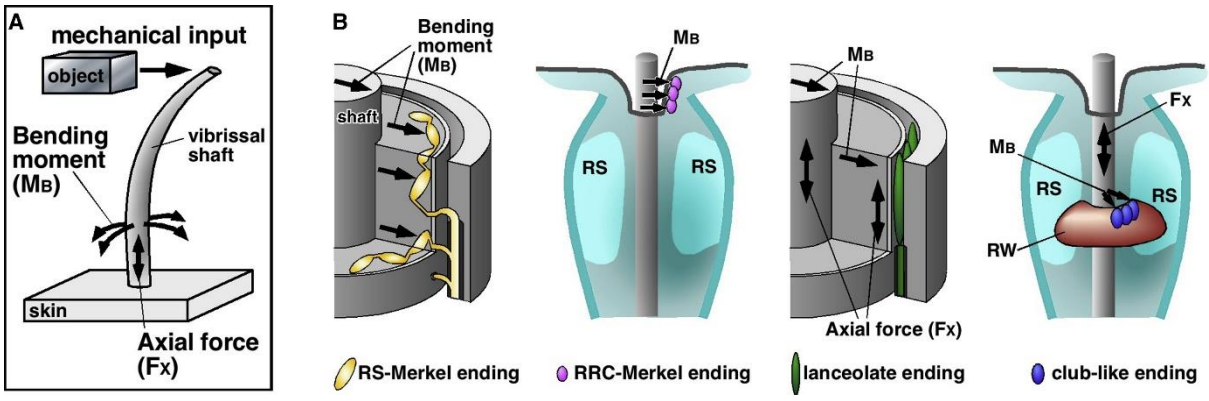


Figure 1-3 **Illustration of the mechanoreceptors in the whisker follicle.** RS – ring sinus, RW – ringwulst. The location of Merkel discs at the whisker follicle is one of the main factors of their response properties: ring sinus, or RS-Merkel endings, are the only slow-adapting

mechanoreceptors and respond the fastest and with the largest magnitude, while rete-ridge collar, or RRC-Merkel discs show longest latency and lowest magnitude of all mechanoreceptor responses and are rapidly-adapting. Adapted from Furuta et al., 2020.

All brain functioning relies on blood flow which is dynamically regulated by vasodilation and constriction to supply glucose and oxygen in support of the neuronal activity: this is the case within the barrel cortex too. This neurovascular coupling is critical for delivering the energy required for processing information (Harris et al., 2012), including the sensory information from whiskers (Cox et al., 1993). Just like the neuronal system, whisker-related vasculature is well defined and shows consistent responses in repeated longitudinal studies, producing reliable measurements in behaving mice, despite variations in locomotion (Takuwa et al., 2011). Recently, it has been suggested as a bi-directional relationship where blood vessels also send feedback signals to influence neural firing (Kumar et al., 2021). Thus, vasculature and neurovascular coupling is important to understand in both health and disease and is especially relevant in Alzheimer's disease. In Chapter 3, I studied the 3xTg-AD model of AD: literature on this model describes early morphological abnormalities and degeneration of microvascular networks, starting from 3 months of age; the onset and progression of microvascular degeneration in this model was cortical subregion-dependent (Quintana et al., 2021). Brain oxidative stress was also shown to occur in the 3xTg-AD mouse model; more specifically, antioxidant levels were decreased at 3-5 months of age (Resende et al., 2008). Any disruptions to the precise regulation of blood flow can cause energy deficits and affect the efficiency of sensory processing and should be investigated in addition to the neuronal, behavioural and muscular functioning.

1.4 Musculature and fascia of the mystacial pad

A lot of work on whisker musculature was done by Haidarliu et al. (2024, 2021 and 2010). They found that whisker motor control relies not only on muscles of the mystacial pad, but also on the surrounding tissues, such as the collagenous skeleton in the snout fascia. In their 2021 study, they found the fascia to be the main tissue type of the mystacial pad, even more common than muscle, maintaining the structure and contributing to whisker movements driven by the muscles discussed in the next paragraph. The three layers of collagenous skeleton – superficial, deep spongy mesh and subcapsular fibrous mat – are interconnected. Superficial and deep spongy layers contain fascial structures that

transmit muscle efforts and are involved in whisking, while the subcapsular fibrous mat keeps whisker follicle spatial arrangement, responds quickly to deformation and connects the mystacial pad to the skull (Figure 1-4; Haidarliu et al., 2021).

The mystacial pad contains intrinsic and extrinsic muscles – intrinsic muscles originate within the mystacial pad, while extrinsic muscles start at the side of the skull and insert themselves into fascial structures of the mystacial pad (in mice: Dörfl, 1982; in rats: Haidarliu et al, 2010, 2024). Intrinsic muscles can be further divided into two types: sling-like intrinsic muscles that protract whiskers, and oblique intrinsic muscles that provide vibrissal torsional rotation (Haidarliu et al., 2010). One sling-like intrinsic muscle connects to one whisker follicle in the same row and were found to control the amplitude and frequency of goal-oriented whisker movements when collecting sensory information (Haidarliu et al, 2024). On the other hand, extrinsic muscles of the mystacial pad insert into the superficial fascial layer and provide whisker protraction, retraction, vertical deflection and signal translation, and thus are divided into protractors, retractors and whisker movers in the dorsal-ventral axis (Haidarliu et al., 2010). Extrinsic muscles also control the trajectories of individual whiskers, i.e., they determine the location of whisker protraction and retraction set points, and shape and position of the mystacial pad, ensuring the whisker direction and search space required (Haidarliu et al., 2024). While intrinsic muscles work to protract whiskers directly, extrinsic muscles need to either pull the superficial fascia layer caudally or pull the subcapsular fibrous mat rostrally to retract whiskers; this involves either nasolabialis and maxillolabialis muscles, or two maxillary parts and internal profunda of the nasolabialis profundus muscle, respectively (Haidarliu et al., 2010). During protraction, extrinsic muscles perform the contraction of two medial parts and the pseudo-intrinsic slips of the nasolabialis profundus muscle, which then triggers the forward movement of superficial fascia layer and distal ends of whisker follicles. Moreover, the vertical spread of whiskers is controlled by the transversus nasi muscle, which moves the dorsal whisker rows (rows A and B in Figure 1-1 and Figure 1-2) upwards, as well as the orbicularis oris part of the buccinator muscle which moves more ventral rows (C, D and E in Figure 1-1 and Figure 1-2) downwards. Alternatively, contraction of oblique intrinsic muscles can also change the vertical spread (Haidarliu et al., 2010).

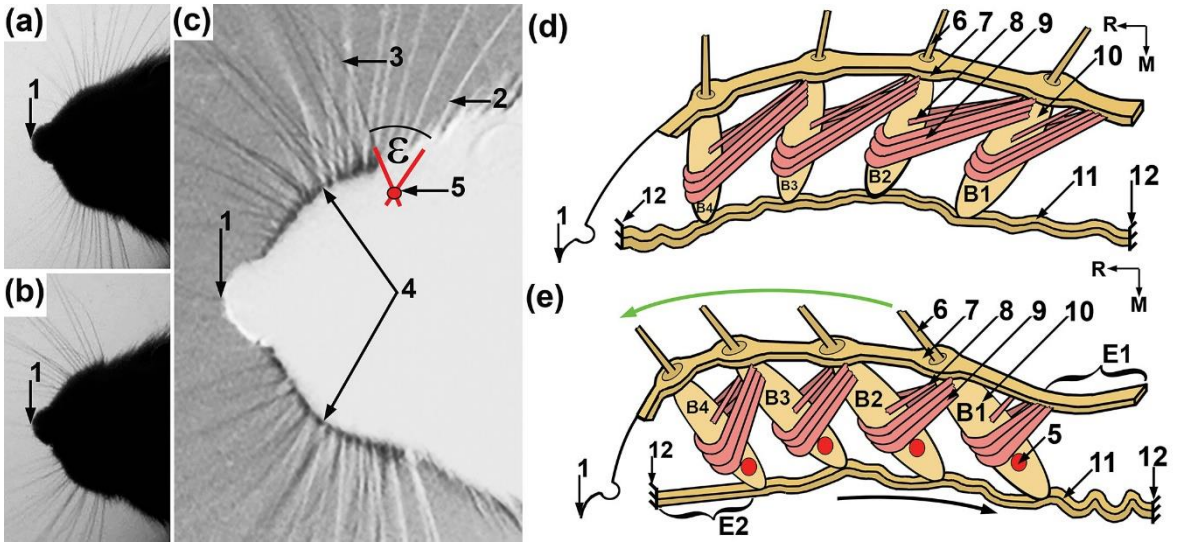


Figure 1-4 **A diagram depicting whisker movement.** Protracted (a) and retracted (b) whiskers are shown. Panel (c) shows an overlaid image of the individual whisker movement, showing one whisker moving from position 2 to 3. Panels (d) and (c) show follicle and muscle positions corresponding to (a) and (b), respectively. (1) Tip of the nose; (2 and 3) retraction and protraction set points, respectively, of the most caudal whisker; (4) rostro-lateral translation of the corium (superficial fascia) of the mystacial pad; (5) pivot point; (6) vibrissa; (7) corium; (8 and 9) oblique and sling-shaped intrinsic muscles, respectively; (10) follicle; (11) subcapsular fibrous mat; (12) anchors of the subcapsular fibrous mat. (ε) Angle of the whisker protraction; (B1–B4) follicles; (E1 and E2) extended parts of the corium and subcapsular fibrous mat, respectively; (M) medial; (R) rostral. Green and black arrows indicate directions of the vibrissae and subcapsular fibrous mat subcapsular fibrous mat movements, respectively; red dots are centers of rotation of follicles (pivot points). Adapted from Haidarliu et al. (2024).

Therefore, for whiskers to move, there is an extensive network of muscle and fascia structures in play. As per Haidarliu et al. (2021), intrinsic muscle force is directly applied to capsules in the follicles. In the fascia, follicles span the superficial and deep spongy layers of collagenous skeleton. Additionally, extrinsic muscle force is applied to structures in the rest of the collagenous skeleton which then transmit the force to the capsules. Protraction and retraction involve the voluntary movement of superficial layer rostrally and caudally by the extrinsic muscles. Finally, upon muscle relaxation, fascial structures return whiskers to the resting position (Haidarliu et al., 2021).

Jin et al., (2004) have found that intrinsic muscles are composed mostly of fast type 2B/2D fibres. In mice, those are the only fibres present, while in rats ~10% of slow type-1 fibres were observed – this is consistent with mice performing faster whisker movements compared to rats. More than 90% of the type 2B/2D fibres in mice and rats correspond to type 2B. Type 2B fibres provide the highest twitch velocity (Bottinelli, 1991; Schiaffino and Reggiani, 1996) and in turn require

copious amounts of energy (Barany, 1967). This is thought to correspond to the need for fast scanning of the environment.

1.5 Rhythmic whisking – a pattern generator drives muscle contraction

Whiskers evolved in many animals as a strategy for efficient exploration of their surroundings, particularly supporting nocturnal animals that cannot rely on their vision at night. The true whisker specialists are rats and mice as they are active in low light conditions and extensively rely on their whiskers. Every step taken by a mouse or a rat falls strictly within the field of their whisker reach (Grant et al., 2018), ensuring there are no obstacles in the way. To achieve this function, whiskers must sweep continuously front and back, protracting and retracting, during exploration. This is called rhythmic whisking, and, in the context of environmental scanning, it consists of large amplitude sweeps. As such, it is also referred to as exploratory whisking (Berg and Kleinfeld, 2003).

For whisking to be a rhythmic event, there is a requirement for a pattern generator in the brain. Moore et al. (2013) found five “whisking units” responsible for this rhythm in the ventral part of the intermediate band of the reticular formation (IRt) and denoted them as the vibrissa zone of the reticular formation (vIRt). The vIRT is in the brainstem, near the preBötzinger complex which generates rhythmic breathing and acts as a master clock to coordinate whisker movements with other mystacial pad and snout movements, such as breathing and sniffing, as well as bilateral whisking (Figure 1-5; Moore et al., 2013, Deschênes et al., 2016). vIRt whisking rhythm generator is unidirectionally connected to the preBötzinger complex, meaning that whisker protractions are driven by the vIRt and time-locked to inspiration, while whisker retractions are caused by the mystacial pad moving in synchrony with expiration, controlled by the Bötzinger/parafacial units (Moore et al., 2013, Deschênes et al., 2016). Additionally, to the five “whisker units” that were active during both inspiratory-locked exploratory and intervening (not associated with breath) whisks, Moore et al. (2013) found 32 units responsible for inspiration and whisker protraction, as well as another 29 units responsible for expiration and whisker retraction. Therefore, whisking is initiated by the five premotor “whisker units” in the vIRt nucleus that activate the intrinsic muscles of the mystacial pad to protract whiskers, and is phase-locked to breathing controlled by the preBötzinger complex; additional inspiratory/protraction units are activated

in the protraction phase, and expiratory/retraction units are activated in the retraction phase (Moore et al., 2013).

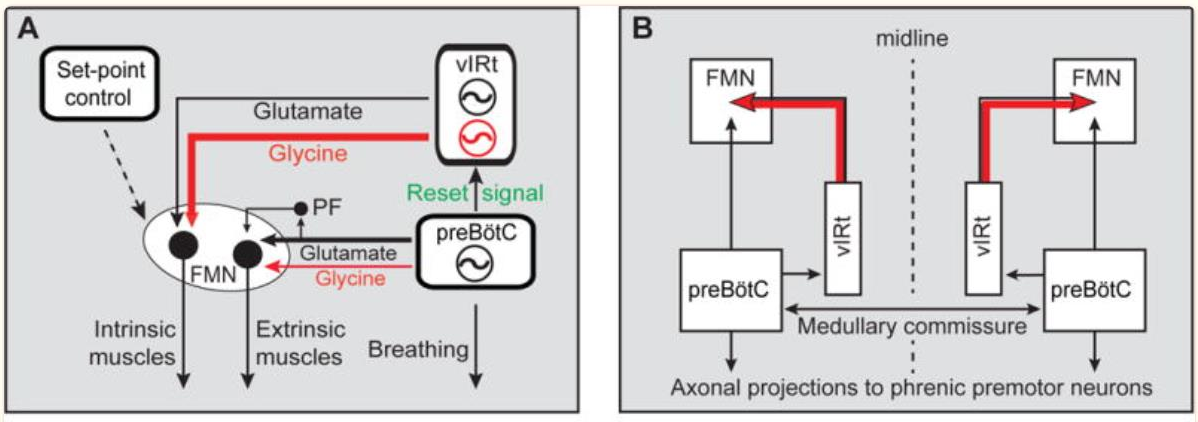


Figure 1-5 **Model of the brain circuits generating the whisker rhythm and coordinating it to breathing centres.** A – central rhythm generator is mostly inhibitory glycinergic/GABAergic neurons innervating intrinsic whisker muscles. B – the whisking reset signal comes from the breathing centre, while the medullary commissure synchronises the breathing centres on both sides of the brain. PF – parafacial neurons, FMN – facial motor nucleus, preBötC - preBötzinger complex. Adapted from Deschênes et al. (2016).

The generation of whisking rhythm is the result of a mainly inhibitory network. The majority of vIRt neurons were identified as parvalbumin expressing inhibitory (mostly glycinergic) neurons (Deschênes et al., 2016, Takatoh et al., 2022). They are the main cells that induce rhythmic whisking; silencing these neurons abolishes whisking completely, causing a sustained whisker protraction (Takatoh et al., 2022). Other types of neurons in the network seem to handle other functions; for instance, Vglut2 expressing (glutamatergic) excitatory neurons are not required for whisking rhythm generation but are likely important for whisking to be coordinated with sniffing (Takatoh et al., 2022). The overall principle of this circuit starts with an initial tonic (slow and persistent) excitation from the facial motor nucleus to the vIRt (Figure 1-6), which in turn activates 28% of vIRt cells to produce an excitatory signal. This drives the intrinsic muscle and whisker protraction. Subsequently, an inhibitory (67% of cells, glycine/GABA) signal from vIRt initiates whisker retraction and supresses the excitatory signal from facial motor nucleus. At the same time, both facial motor nucleus and vIRt receive an inhibitory reset signal from the preBötzinger complex to coordinate whisking with breathing (Deschênes et al., 2016, Takatoh et al., 2022). Moreover, locally in the vIRt, there seems to be a dense network of reciprocal inhibitory synapses, likely forming a recurrent network, as is common in other rhythmic systems (Marder and

Bucher, 2001, Takatoh et al., 2022). Individual cells in vIRt do not behave cyclically at rest, but rather, the rhythm is generated as a property of the network and depends on the initial external input as well as the recurrent local inhibition (Takatoh et al., 2022).

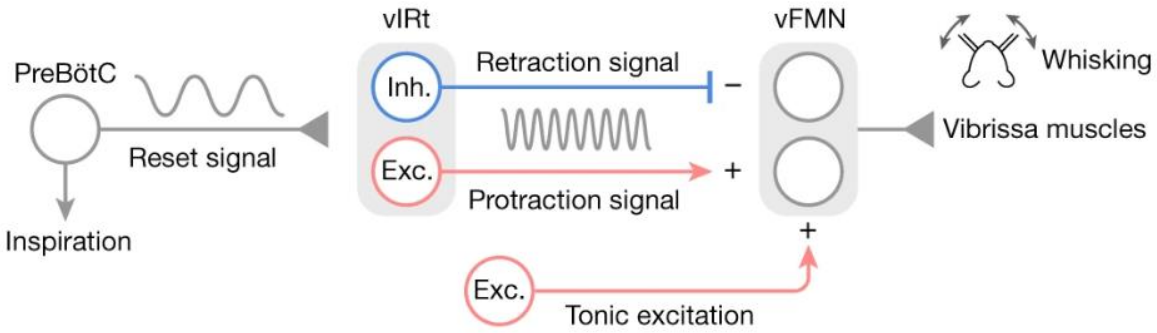


Figure 1-6 **Whisking rhythm generator circuit in the brainstem.** The generation of whisking rhythm is the result of a mainly inhibitory network. The circuit starts with an initial tonic excitation from the facial motor nucleus to the vIRt, which activates vIRt to produce an excitatory signal driving the intrinsic muscle and whisker protraction. Then, an inhibitory signal from vIRt initiates whisker retraction and supresses the excitatory signal from facial motor nucleus. At the same time, both facial motor nucleus and vIRt receive an inhibitory reset signal from the preBötzinger complex to coordinate whisking with breathing. Exc. – excitatory, Inh. – inhibitory, vFMN - vibrissal part of the facial motor nucleus. Adapted from Takatoh et al. (2022).

1.6 The function of whisker movements – acquisition of sensory information and higher executive functions

Rhythmic whisking is an advantageous strategy to actively acquire lots of sensory information. The amplitude and frequency of whisking is controlled in rats and mice. It provides information about the timing (onset, duration, termination, repetition), peripheral location (deflection direction, amplitude of movement) and speed of the stimulus. Depending on the goal, exploratory whisking can be employed to actively scan the environment by protracting and retracting whiskers at lower frequencies (7-12 Hz in a rat) and larger amplitude movements (Berg and Kleinfeld, 2003). Upon encountering an object, rodents may switch to foveal whisking, where protractions occur at higher frequencies (15-25 Hz in a rat) and smaller amplitudes, while retraction is a slower and more passive process. This allows gentle palpation around an object to maximise the information collected (Berg and Kleinfeld, 2003; Grant et al., 2009).

Whisking as a behaviour does not exist on its own. It is closely related to other activities essential to survival and even to some higher executive functions. Rhythmic whisking is coupled to breathing, and, therefore, whisking is coordinated with other behaviours such as sniffing, licking, suckling and chewing (Moore et al., 2013; Ito et al., 2014; Kleinfeld et al., 2014). Because of the primary function in navigation, whisker movements have a direct impact on foraging (Anjum et al., 2012; Muchlinski et al, 2018; Grant et al., 2021), pups' feeding (Sullivan et al., 2003), and social contexts (Wolfe et al., 2011). For instance, bilateral whisker trimming in early life has been found to reduce brain oxytocin levels and impact social discrimination and social memory in adult male mice, but not object memory (Pan et al., 2022). Whisking is also coordinated with head movements; in fact, whisker movements predict the decision of the direction of turn better than eye movements (Towal and Hartmann, 2006; Bergmann et al., 2022). Additionally, the condition of whiskers is evaluated as part of the rodent grimace scale used for evaluating pain and wellbeing (Langford et al., 2010; Sotocinal et al., 2011). Whiskers are also a part of the facial expression and are correlated with emotional reactions to fear of threat and noxious stimuli (Elbaz et al., 2022). Bernhard et al. (2020) report sensory association learning to be whisker-dependent, suggesting a role in memory. Moreover, rodents actively pay attention to different environmental settings, and, in response, they change strategies of whisker touch to maximise sensory information collected (Arkley et al., 2014), offering a way to study attention without the need for stressful external motivators such as fear or food restriction. Whisker movements also guide locomotion. Grant et al. (2018) found that whiskers tend to always scan ahead of where the forefeet were positioned on the floor during forward locomotion on a flat floor, as well as during climbing (Arkley et al. 2017).

A completely new type of exploratory whisking emerges as soon as the rodent contacts an object. When touching an object, rodents tend to reduce their whisker spread, making the gaps between whiskers smaller, which is thought to increase the number of whisker contacts against a surface (Grant et al., 2009; Figure 4 in Simanaviciute et al., 2020). Once the whiskers contact a surface, they tend to decrease their retraction speeds, which is thought to increase the amount of time in contact with the object (Grant et al., 2009). The whiskers can also contact the object asymmetrically, a process termed contact-induced asymmetry. This helps to maximise touches with object, especially seen on the whisker side opposite to the

object (contralateral), which make large amplitude movements and are positioned far forward with larger angles. It also enables light touches, since the whiskers on the contacting side (ipsilateral to the object) gently palpate the object with low amplitude movements and small whisker angles (Mitchinson, 2007, Grant et al., 2012). Indeed, this balance of behaviours is termed ‘minimal impingement, maximal contact’ – whisker control serves to increase the time and number of whiskers in contact with an object, whilst at the same time enabling light, clear touches. From the positioning and focussing of the rat’s whiskers onto the object, we may also infer their attention (Mitchinson and Prescott, 2013), which is a goal-oriented behaviour and offers us a link to rats’ cognitive abilities.

When a rat contacts an object, they may also increase the frequency of whisking, which changes the activation of both intrinsic and extrinsic muscle types to anti-phase (Berg et al. 2003). However, this behaviour is not often observed, as frequency of whisking is different in rats and mice and can have multiple first order and, at times, second order harmonics (Mitchinson et al., 2011), hence, becoming difficult to model and approximate. Therefore, in my studies, frequency will not be evaluated due to how highly variable it can be and how difficult it is to reliably quantify. Moreover, this is one of the many reasons why pre-contact and during-contact values will be assessed separately.

In addition to object-related exploratory whisking, several other whisker behaviours are used to optimise navigation and orientation. When locomoting at high speeds with a risk of collision, a “look-ahead” behaviour is sometimes employed, meaning whiskers are highly protracted and whisking amplitude is decreased (Towal and Hartmann, 2006; Arkley et al., 2014). When the rodent is about to turn its head, whisker movements will precede and indicate the side of the turn by retracting to the same side - another whisker behaviour called ‘head turning asymmetry’ (Towal and Hartmann, 2006; Mitchinson et al., 2011). These behaviours may reveal spatial attention, especially focussing the whiskers onto an area that the animal is about to move into (Arkley et al. 2014).

1.7 Methods of studying whisker movements

As whiskers have both sensory and motor aspects, there are many ways of studying them. For the sensory aspect, a neural recording is often the choice. Ever since 1969, researchers have been performing electrophysiology studies to

assess what stimuli whiskers respond to by studying trimmed single-whiskers in head-fixed, anaesthetised rats (Zucker and Welker, 1969). Neural imaging has also been utilised: two-dimensional optical imaging spectroscopy recordings can be collected during mechanical whisker stimulation (Sharp et al., 2015). Similarly, functional magnetic resonance imaging recordings using air puff as a stimulus can be collected from the thalamus (Sanganahalli et al., 2022) and from the barrel cortex during whisker deflection (Lu et al., 2016).

There have been advances in naturalistic behaviour tracking and the combination of that data with invasive neuronal recordings to discover grid cells (Hafting et al., 2005), and, even quite a bit earlier, the discovery of place cells (O'Keefe and Dostrovsky, 1971). Considering locomotion and head movements are important, studies that focus on neural recordings are likely to benefit the most from the newest electrophysiological recording tools such as neuropixels (Steinmetz et al., 2021). A significant limitation of this technique is that once the implant is in the mouse brain, it cannot be removed. Supposedly, it being lighter than 10% of the mouse's body mass means that the mouse is not constrained by it, however, it may impact still their behaviour. We want to be able study freely moving, naturally-locomoting animals and connect that to sensorimotor function; however, ways of doing that are currently limited and studying whiskers could be the way forward.

More recently, specialty approaches using the newest techniques in computer vision and deep learning have been developed, which might also be interesting to adapt to study whisking. Klibaite et al. (2022) used such approach to analyse data collected in an open field for the study of repetitive behaviours in a mouse model of autism spectrum disorders. However, this has not yet been related to whisking, despite it being essential to rodent's survival and having major impact on the locomotion measures that are often estimated from pose in these machine learning methods.

1.8 The challenges of developing behavioural tasks

To model human disease in laboratory animals and test all the potential symptoms of that disease, researchers have been moving from single behavioural tests to test batteries, which are more extensive tests on sensory and motor functions (Saré et al., 2021). Some of the gold standard behavioural tasks that are combined and introduced to animals in an increasing level of invasiveness include

locomotion tests in the open field, rotarod, various mazes to assess anxiety levels, spatial learning test in the Morris water maze and many types of memory-focused tasks such as the novel object recognition paradigm (Pellow et al. (1985); Nicolas et al. (2006); Prut and Belzung, (2003); Asinof and Paine (2014); D'Hooge and De Deyn, (2001); Antunes and Biala (2012); Crijns and Op de Beeck (2019), Table 1-1). The more complicated the experimental question, the more controls must be introduced, and often that means habituating and training animals to perform many tasks, which takes a long time. Similarly, the more training and habituation is required, the more skilled the animal handler needs to be, and the more day-to-day variability is introduced by different handlers (Georgiou et al., 2022) as well as environmental, hormonal and other expected or unforeseen factors, which are extremely difficult to control for. It is important to consider that some animals will never be able to perform above chance in the more complex tasks, meaning that the sample size must be increased to account for the dropouts (which will be further discussed in Chapter 6 Discussion), resulting in more animals being used in tests. Furthermore, the data recorded tend to be either simple durations, percentages or counts of correct choices (see Table 1-1, Main measurements column), which are not highly quantitative, and may also require larger sample sizes for the analysis to be powerful enough.

Especially for the study of cognitive functioning, it can be difficult to develop a rodent task that specifically tests the one intended aspect of interest. For instance, the standard novel object recognition task can be used to assess the effect of environmental distraction on memory. However, one must change this task into the **continuous** novel object recognition (Ameen-Ali et al., 2012) to separate the effects of distraction from those of proactive interference, which is when earlier memories disrupt the formation of new memories (Landreth et al., 2021). Both distraction and proactive interference are symptoms of memory-affecting diseases such as schizophrenia and Alzheimer's disease, therefore, they must be assessed separately when looking for treatments. Likewise, when studying important aspects of executive functioning, we are not purely interested in one type of memory; we may wish to test strategy, planning, decision-making, attention, behavioural flexibility, problem solving, reaction time and choice accuracy, all aspects of cognition that can be affected by disease without visible memory deficits. To reliably test these, the experimenter must include not just the standard but also reversal tasks, as well as keep changing the goal of the task and re-

training the animals. Consequently, one might wonder whether it is useful to continue with such tasks, or if a simpler approach with better quantification of behaviour and automated techniques could be useful.

In some tasks, animals were found to perform differently depending on their sex and hormonal status. An example of that is the fear conditioning test, where proestrus female rats have shorter freezing durations and longer locomotion distances, compared to male and dioestrus female rats (Pyeon et al., 2023). Similarly, female mice were found to perform better at the novel object recognition task under stress conditions (Torrisi et al., 2023), while males can perform better in spatial orientation tasks such as a water maze (Melgar-Locatelli et al., 2024).

Especially relevant for this thesis is the challenge presented by whisker trimming and barbering in the performance of behavioural tasks. As whiskers are essential in many motor, sensory and executive functions, is it not surprising that classic behavioural tests such as the open field, novel object recognition and marble burying can all be impacted by full de-whiskering or partial whisker desensitization in the C57BL/6 mouse strain, commonly used as control animals (Haridas et al., 2018). Missing the inspection of whiskers before any behavioural task can thus lead to inaccurate results, as whisker barbering is a rather common way to establish hierarchy in laboratory rodents, especially in mice housed in groups (Sarna et al., 2000).

Home cage systems (such as IntelliCage by Endo et al., 2011) offer a way to observe the animals non-invasively in their home environment, thus reducing the stress of removing them from their usual cage. However, sometimes training is still required for tasks performed in IntelliCage; otherwise, it can only provide more general locomotive and social measure. Indeed, they often don‘t give the same level of fine-scale movement and quantification of behaviour as whisker movement measures. However, home cage monitoring systems are constantly and consistently being developed and may be an interesting avenue of research in the future. One thing that home cages systems tend to offer is a full automation of the tasks and data analysis (Endo et al., 2011). Automation is likely to reduce variability and subjectivity in behavioural testing and may increase reproducibility of results by keeping caging and management systems common across different labs.

Table 1-1 **Summary of a selection of several commonly used behavioural tasks.** All tasks in the table have significant drawbacks related to restriction of movement, being invasive or stress-inducing, requiring training of extensive habituation, or providing measures with a lower level of granularity.

Task domain	Task subdomain	Tasks	Main measurements	Drawbacks	Reference
Anxiety/fear/avoidance	Anxiety-like behaviour	Elevated plus maze	Locomotion distance, time spent in the open arms, latency to enter open arms	Requires habituation, stressful	Pellow et al. (1985)
	Repetitive, compulsory-like behaviour	Marble burying	Percentage of buried area	General measures do not capture highly quantitative behaviour	Nicolas et al. (2006)
	Anxiety, motor deficits	Open field	Locomotion distance, time spent in the centre zone, time spent on behaviours of interest such as rearing or grooming	General measures do not capture highly quantitative behaviour, unclear if really measures anxiety	Prut and Belzung, (2003)
Cognitive/executive	Attentional	5-choice-serial reaction time	Correct response ratio, speed of response and reward retrieval, number of nose pokes	Requires training, not highly quantitative	Asinof and Paine (2014)
	Spatial learning	Morris Water maze	Latency to reach the platform, total path length to platform	Requires training, highly stressful	D'Hooge and De Deyn, (2001)

	Novelty / oddity recognition	Novel object / place	Time of object exploration (nose/paw touching or nose pointing towards and within 2 cm of an object) as a ratio of (time exploring the novel object minus the time exploring the familiar object / location) divided by the total time exploring both objects	Requires multiple habituation sessions for sampling, sometimes requires food restriction to encourage exploration	Antunes and Biala (2012)
Sensory	Visual	Touchscreen for visual discrimination	Percentage of correct responses is used to determine the degree of visual acuity	Requires habituation and training, food restriction	Crijns and Op de Beeck (2019)

1.9 Standardisation of behavioural tasks

Many scientific theories have been called into question due to an ongoing reproducibility crisis, a methodological issue where experimental results of a study cannot be replicated (Ioannidis, 2005). It arises from natural variation in husbandry practices and behavioural tasks between laboratories, where even having different experimenters can affect rodent behaviour (Crabbe et al., 1999; Georgiou et al., 2022). To increase internal and external validity across studies, it is important to automate tasks as much as possible (Krackow et al., 2010). Making tests quick and reducing day to day variability are all important factors too. Including general activity and behavioural measures over longer periods of time, such as home-cage monitoring, might also be useful for behavioural tasks.

Overall, there is a need for standardisation to make animal experimentation as robust as possible and reduce variation. In the first instance, this includes strict reporting of methodological details of husbandry practices and detailed experimental methods, including describing environmental factors that may affect

experiments, such as noise and light levels. Sharing data to allow for easy comparisons between laboratories is also important. Considering the factors that may lead to variation in experiments and impact research reproducibility is an important first step in designing robust behavioural tasks.

Amongst many aspects of the reproducibility crisis, the 1980's shift from rat to mouse studies revealed that the behavioural differences between the two most widely used animal species in research are not to be overlooked. Additionally, there are large behavioural differences between mouse background strains, also confirmed to be the case in my previous whisker movement study (Simanaviciute et al., 2020). Any type of behavioural testing can be affected by animal handling and the experimenter, suggesting that even the most carefully controlled experiments might produce different results. The ideal behavioural study would likely include home cage monitoring as a baseline for measuring anxiety-free behaviour, and even construct a testing system by connecting it to the home cage (Voikar, 2020).

Indeed, developing robust, quantifiable measures of behaviour may also reduce variation and increase behavioural standardisation. The importance of extensive behavioural tests, including the quantification of sensory abilities and motor functions, open field locomotion, as well as monitoring of whisker appearance and whisker reflex, has been emphasised for a very long time as the main methods for reducing false positives and false negatives (Crawley, 1999). This list is quite encouraging to see, since whiskers are a non-standard method of studying behaviour, but they do fit quite well with these recommendations.

The current battery of tests used for assessing rodent behaviour consists of either expensive and intrusive methods or requires extensive animal training. They also result in only simple, low granularity behavioural measures, such as counts, durations or frequencies. I propose that measuring whisker-related exploratory movements offers an alternative way to observe highly quantitative behavioural changes in mice. Not only does it measure an intrinsically motivated exploratory behaviour which should enable universal measures despite the disease symptoms, but also takes minimal time and experimenter experience as it does not require animal training or habituation. Hence, video clip collection from animals is much faster than from habituation and training requiring tests, and there is minimal day-to-day environmental variation such as changes in handlers, noise

and light levels, allowing the data to be more consistent. The low environmental variation for this method creates suitable conditions for the whisker tracking to be semi-automated today, and potentially fully automated in the future, which could speed up result collection even more. Minimal handling is also less stressful to animals than training which could take months of daily handling. This truly means minimal invasiveness and disturbance, as well as compliance with the 3Rs, especially in the refinement and reduction of animals. In fact, this method could be integrated with, or used in tandem with, other laboratory tests as it is comparatively easy to introduce the animals to, and it does not interfere with other behavioural tests, especially when done in parallel. Because this approach can work well with other behavioural tests and allows to measure sensory, motor and even some cognitive functions, it may provide the added benefit of reducing the overall number of animals used in the experiments by increasing the amount of data collected from each animal and validating the results seen in other behaviour tasks, avoiding using larger sample sizes just to reach statistically significant findings.

1.10 Studying disease in the whisker system

Considering whisker movements are directly related to the facial movements, whisker tracking is particularly useful in facial nerve palsy research (Takahiro et al., 2023) and trigeminal neuropathic pain studies (Koizumi et al., 2021). Similarly, the whisker pad has been suggested as a model system for cutaneous squamous cell carcinoma of the head and neck (de Lima et al., 2023), and cancer-induced pain (Gutierrez et al., 2021). Furthermore, the whisker system has been used as a model for studying functional hyperaemia - the activity-dependent increases in local blood perfusion (Ferris et al., 2023) - and is also a well-established model for neurovascular coupling studies (Zehendner et al., 2013; Kennerley et al., 2012). Perhaps unexpectedly, whisker follicles have been used to study gene expression in methamphetamine use disorder models (Jang et al., 2020). However, most diseases studied in the whisker system are neurological; therefore, the first versions of the protocol used in this thesis were also tested in mouse models of neurological disease. Findings from whisker movement studies in those disease models showed that:

1) SOD1 mice, a model of Amyotrophic lateral sclerosis, showed whisker movement differences at postnatal day 60 (~8 weeks, Grant et al., 2014);
2) R6/2 (CAG250), zQ175 and Hdh (CAG250) mouse models of Huntington's disease showed whisker movement differences at 10 weeks of age (Garland et al., 2018);
3) 5xFAD mice, a model of Alzheimer's disease, showed whisker movement differences at 6 months of age (~ 26 weeks, Grant et al., 2018).

These studies were the first to demonstrate the potential of using this whisker tracking protocol in mouse models of neurological diseases affecting sensorimotor and executive functions. They also demonstrated the contact-related changes in whisker movements which are important to this protocol and will be presented in Chapter 2 General methods. Such changes are reproducible robust behaviours, and their presence or absence must be considered in studies where treatment effects are investigated. I will be referring to these behaviours throughout my experimental chapters (Chapters 3-5).

1.11 My previous contributions to the field

I started out working in this field during my undergraduate placement year, when I contributed to the development of the automated rodent tracker ART, especially in its testing phase. This work was published in the Journal of Neuroscience Methods 328, entitled "Description and validation of the LocoWhisk system: Quantifying rodent exploratory, sensory and motor behaviours" by Gillespie, D., Yap, M.H., Hewitt, B.M., Driscoll, H., Simanaviciute, U., Hodson-Tole, E.F., Grant, R.A., 2019.

The tracker was then used in all my further studies, starting with my main work from the placement year, where I found differences in whisker movements in 8 different mouse models when compared to control mice. This work was published in Journal of Neuroscience Methods 331, entitled "Recommendations for measuring whisker movements and locomotion in mice with sensory, motor and cognitive deficits" by Simanaviciute, U., Ahmed, J., Brown, R.E., Connor-Robson, N., Farr, T.D., Fertan, E., Gambles, N., Garland, H., Morton, A.J., Staiger, J.F., Skillings, E.A., Trueman, R.C., Wade-Martins, R., Wood, N.I., Wong, A.A., Grant, R.A., 2020. In the article, I made recommendations for whisker tracking producing

the schema (Figure 8 in Simanaviciute et al., 2020) which is used as a starting point for this thesis.

I later continued working with the tracker and whisker protocol for my final undergraduate degree project and, for the first time, tested it in a rat model of schizophrenia. No differences in whisker movements in rats were found, but that conclusion supported the findings of the memory deficit in treated rats. This study was published in Brain and Neuroscience Advances 5, entitled “Dissociating the effects of distraction and proactive interference on object memory through tests of novelty preference”, by Landreth, K.*, Simanaviciute, U.*, Fletcher, J., Grayson, B., Grant, R.A., Harte, M.H., Gigg, J., 2021 (* - equal first author contribution) and highlighted the benefits of incorporating whisker measurements with other behavioural tests as well as introduced further questions about when adopting the whisker protocol is the most beneficial. These are important questions that I develop here in my PhD thesis.

Within this thesis I have developed the ARTv2 tracker to include a new quantitative measurement of whisker spread. In Chapter 3, I describe how all quantitative measures from ARTv2 tracker were validated against previous manual qualitative scoring of whisker movements. This resulted in all qualitative scoring being replaced by the quantitative measures produced by the semi-automated ARTv2 tracker.

1.12 Areas to develop to improve the adoption of whisker behaviour for the study of rodent neurological symptoms

At the beginning of this project, I proposed that measuring whisker-related exploratory movements could offer an alternative way to observe highly quantitative behavioural changes in mice. I had previously demonstrated this by assessing whisker movement differences in mouse models of a wide range of neurological disorders. However, whisker movements had never been studied in tandem, nor compared to, other established laboratory tests. Some aspects of the protocol were still manual (Figure 1-7, the two qualitative scoring steps in the open field and the object exploration task) and would have benefited from automation. The protocol thus far had only shown significant whisker movement differences in mice; therefore, I also needed to show whether it could reveal whisker movement deficits in other species, specifically, rats. The protocol had been used in studies

that included male and female mice (Grant et al., 2018, Simanaviciute et al., 2020) and sex was shown to affect whisker movements. Therefore, I suggested that both male and female animals should be included where possible and further statistical considerations might need to be applied when using the protocol. In addition, this method had only been validated in open field and in one type of novel object exploration task. To be more adaptable to many different disease models and laboratory set ups, this method needed to integrate with other types of tasks and provide additional general locomotor-exploratory measures. These were missing at the time as the focus was mostly placed on high-resolution whisker movements without the context of the general animal movement and behaviour.

Questions that especially needed to be addressed included:

- How could we move towards a standard test of whisker movements?
- We did not always see differences in whisker movements, so which models would I recommend to study?
- How did whisker movement fit with other behavioural tests?

1.13 Thesis aims and objectives

In this thesis, I will make recommendations for the application of whisker movement measures for the study of rodent models of neurological disease; focussing on elements that I have drawn out my literature review, including:

i) Standardisation
Especially adding a quantifiable whisker spread measure to the normal measurements and standardising statistical methods for all future studies.

ii) Automation
By removing and replacing elements of manual qualitative scoring, presented in the first two steps of the workflow in Figure 1-7.

iii) Integration
Integrating whisker movements with other behavioural tasks and placing them within the context of general exploratory behaviour.

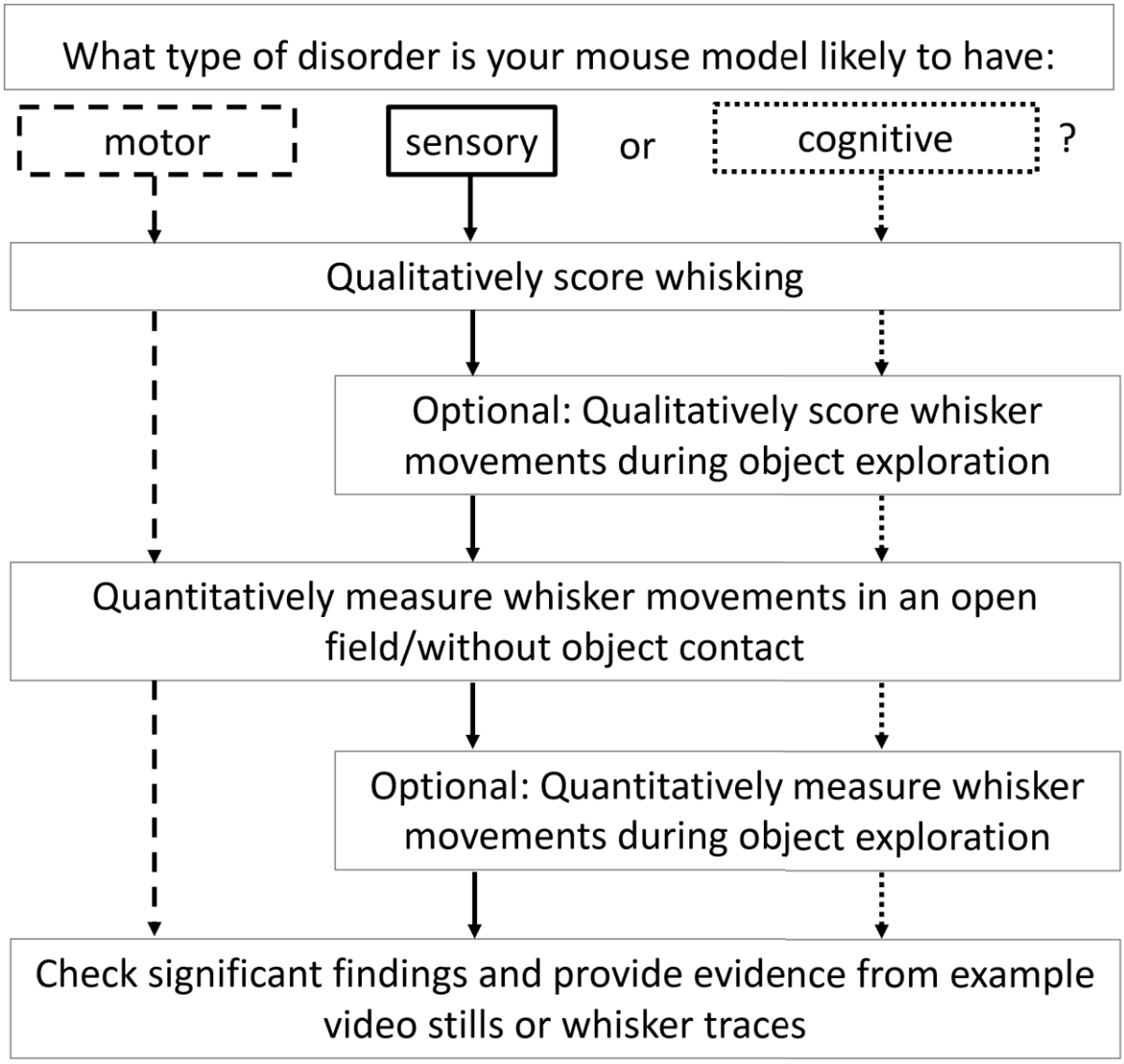


Figure 1-7 **The current** (based on Simanaviciute et al., 2020) **recommendations for whisker tracking, depending on the symptoms exhibited.** Includes qualitative (manual) and quantitative (automatic) measures. The open field test is suggested as the standard, while the novel object exploration test is currently recommended as an optional test for revealing sensory and cognitive deficits.

1.14 Structure of this thesis

My thesis will consist of three experimental chapters, using three rodent models, including:

Chapter 3: 3x-AD mice

The triple transgenic (3xTg-AD) mouse model of Alzheimer's Disease is important in biomedical research as these mice develop both neuropathological and behavioural phenotypes. However, their behavioural phenotype is variable, with

findings depending on the specific task, as well as the age and sex of the mice. I examined whisker movements in 3, 12.5 and 17-month-old female 3xTg-AD mice and their B6129S/F2 wildtype controls.

Chapter 4: MIA rats

The maternal immune activation (MIA) rat, induced by an environmental risk factor polyinosinic:polycytidylic acid (Poly I:C), is a model of neurodevelopmental disorders. Their behavioural symptoms are diverse; they are often variable and reduce in severity or disappear when offspring reach adulthood. Measuring whisker movements presents an opportunity to study innate exploratory rodent behaviour in these animals, resulting in highly quantitative, robust measurements of sensory, motor and cognitive behaviours. However, measuring whisker movements has not yet been proven to work in rat models. In this study, I investigated whisker movements in adult male and female offspring of MIA-exposed rat dams and compared to age-matched offspring of control (vehicle) dams.

Chapter 5: Reeler mice

Reeler mice have been suggested as a model for several disorders, including the developmental disruption of cortical layers, lissencephaly type 2 and perhaps even epilepsy. Homozygous reeler mice have a complex behavioural phenotype and few studies have revealed robust behavioural and cognitive deficits in these mice, despite their neuroanatomical disruptions. Using the standard whisker protocol, only one whisker measure – pre-contact spread - was found to be affected by genotype. Further testing revealed that previous exposure to the arena might influence whisker measures obtained using the standard protocol. Additional testing was performed where whisker movements were filmed in an open field environment and during a further habituation period of five sessions.

Chapter 6: Discussion

My findings and observations from these three studies will come together in the discussion chapter of my thesis. I will make recommendations for future work and present a workflow for robustly and repeatedly studying whisker movement measures in rodent models.

CHAPTER 2 GENERAL METHODS

2.1 Data collection

During my PhD studies, I travelled to three different animal facilities at Dalhousie University in Halifax (Canada), the University of Manchester in Manchester (UK), and the University of Göttingen in Göttingen (Germany), to setup the same equipment in all those laboratories and run my experiments. The studies were run within the animal facilities and animals were not moved outside of those facilities; however, I did not handle the animals myself and was not allowed inside the housing facilities due to local licensing and animal welfare rules. Furthermore, as I was a short-term visitor at these facilities, I had no control of the animals available to me, which is why Chapter 3 only includes female mice.

For the work described in this thesis, Chapters 4 and 5 contain data which are the direct results of my travels and my own data collection in the UK and Germany, respectively. While I did travel to the same laboratory at Dalhousie University to collect similar data for a different mouse model not included in this thesis, the dataset used in Chapter 3 was collected prior by my PhD supervisor using the same method.

Due to the limited time available at each of the institutions I travelled to, the focus was on setting up the experiment and collecting the data. While the original ARRIVE guidelines from 2010 (Kilkenny et al., 2010) stated that details on bedding material should be reported, the data in this thesis were collected between 2016 and 2021 and, at that time at the facilities I was visiting, recording information on handling and bedding practices was not yet commonplace. Cupping (mostly mice) and handling by the scruff (mostly rats) was used, while tail handling was not used. In all studies, nesting material was provided, such as soft-wood shavings. However, the exact brands and quantities were not recorded, and I am not able to provide the exact information in this thesis, nor to compare them between the facilities I visited. In Chapter 6 Discussion, I recommend details on animal handling and bedding are reported in future behavioural studies.

With the publication of the ARRIVE 2.0 guidelines in July 2020 (Sert et al., 2020), the importance of why and an example of how such information should be reported were provided. In support of my observations, the authors of ARRIVE 2.0

also admit the impact of the first edition was limited and a revision was necessary to make it easier for researchers to adopt. To improve the quality, reproducibility and translatability of animal studies, it is not only important to follow ARRIVE reporting guidelines but also the more recent complementary PREPARE guidelines for study planning (Smith et al., 2018). Hence, in Chapter 6 Discussion, I recommend both ARRIVE (last step in Section 6.1 Findings – The protocol) and PREPARE (pre-step in Section 6.1 Findings – The protocol) guidelines are followed in all future animal studies.

For filming whisker movements, animals were placed in a transparent Perspex rectangular arena (30 × 50 × 15 cm) which was lit from below by an infra-red light box (LEDW-BL-400/200-SLLUB-Q-1R-24 V, PHLOX) in Chapters 3 and 5, or by a visible white light box (60 cm × 85 cm, MiniSun LightPad). The lights used did not produce any observable temperature changes, although this was not measured and may be a potential confound when compared between the beginning and the end of the experiment. The rectangular arena was cleaned with a 70% ethanol spray after each animal to remove any olfactory cues. Once in the arena, animals were filmed from above using a digital high-speed video camera (Phantom Miro ex2) recording at 500 frames per second with a shutter-speed of 1 ms and resolution of 640 × 480 pixels. Multiple 1.6 s video clips (800 frames) were collected opportunistically (by manual trigger) when the animal moved into the camera's field of view. The aim was to collect 10 to 15 videos per animal. However, animals were removed from the testing arena after 15 min, independently of the number of videos collected.

During the video collection for the study described in Chapter 5, I noticed for the first time that the animals were distracted by the experimenter in the room, hence, the sides of the arena were covered in light brown paper. It may be beneficial to choose opaque sides for the testing arena. Existing literature suggested that mice and rats perceive red colour as dark, making it a logical choice for this setup.

However, the specific colour choice may require further experimentation: for example, Gjendal et al. (2018) state that mice prefer not to stay in coloured shelters, and when they do choose, blue and amber colours are preferred over red. As my setup aims to encourage exploration, it is not clear if red arena walls would be more appropriate than, for example, blue.

2.2 Video processing

Video clips were selected for qualitative (Chapter 3 only) analysis based on the criteria developed by Grant et al. (2014). These criteria were: i) the head of the animal was clearly in the frame; ii) both sides of the face were visible; and iii) the head was level with the floor (no extreme pitch or yaw).

For quantitative analysis (Chapters 3 to 5) of whisker movements and locomotion in tasks involving objects, video clips were manually divided into pre-contact (PC) and during object contact (DC). Therefore, the clip selection criteria were amended to also include i) the animal must be travelling toward the object in the PC section of the clip; and ii) the whiskers were only contacting the object and not the vertical arena walls, in the DC section of the clip. In this way, general whisker movements could be assessed for motor behaviour in the PC section of the clip (like an open field), and object exploration could be assessed in the DC section of the clip. Only clips that had both considerable PC and DC segments (>0.2 s) were included in this quantitative analysis. The clips were tracked using the Automated Rodent Tracker, version 2 (ARTv2), which was validated as comparable in accuracy to other software and manual trackers on the market (Gillespie et al., 2019). This used image processing to automatically locate the snout, for whisker parameters, and the centroid of the animal, for *locomotion speed* calculations. A ruler was filmed at the start of each episode of data collection to enable a calibrated measure of locomotion speed in metres per second.

The whisker detector program (ARTv2) automatically found the orientation and position of the snout, and the whisker angles (relative to the midline of the head) of each identified whisker. The ARTv2 program is only able to detect whiskers and does not maintain the identity of the whisker between frames (i.e., tracking); rather, a mean angle is calculated from each frame using all detected whiskers. Larger whisker angles represent more forward-positioned whiskers. If whiskers are occluded (such as by whisker crossing) the software will not detect them; therefore, the number of whiskers detected can vary from frame to frame, with a total of 2–12 whiskers detected in each frame (with around 10–12 whiskers being usual, 5–6 on each side). Whisker detection was validated by manually inspecting the software annotations overlaid onto the video frames.

Our laboratory has been developing whisker trackers for many years: it started with a manual whisker tracker (Hewitt et al., 2016), then an automated rodent

head and body tracker (ART, Hewitt et al., 2018), and finally produced ARTv2 which tracks both body and whisker movements and was used in this thesis.

At the time of ARTv2's development, there were some trackers available, but they did not fit the needs of this experimental setup. Many of these existing trackers were, and still are, either setup for head-fixed, close-up whisker movements with no background changes, and not optimised for object contact (Whisk developed by Clack et al., 2012; WhiskEras developed by Betting et al., 2020; an automated homecage system developed by Bernhard et al., 2020; DeepLabCut used by Sehara et al., 2021) or tuned for body tracking and are thus not as accurate at tracking whiskers (EthoVision, Spink et al., 2001). My videos capture body and whisker movements, and as such needed a tracker which could handle both simultaneously.

When ARTv2 was in development, BWTT (Percon et al., 2011) was the best tracker available. However, it relied on a MATLAB interface which is not used by evey laboratory, and personal observations had shown that BWTT does not work as well when tracking whiskers contacting objects, which is a crucial part of my experiments. As part of ARTv2 development, it was validated against both BWTT and Whisk - ARTv2 was found to perform as well as BWTT and significantly better than Whisk (Gillespie et al., 2019). Overall, the open-source nature and the functionalities of the ARTv2 tracker made it the obvious choice for my studies. It is worth noting that these considerations do not mean that deep-learning methods such as DeepLabCut could not be used to train a neural network to work in studies such as mine.

2.3 Whisker parameters

Mean whisker angle was calculated by taking the mean of all the detected whiskers on each side, on a frame-by-frame basis.The following variables were then calculated from the mean whisker angles: *mean angular position* (the average whisker angle), *amplitude* ($2\sqrt{2}$* the standard deviation of whisker angles, to approximate the range of whisker movements), *asymmetry* (the difference in whisker angles between the left and right sides), and the *mean angular retraction* and *protraction speeds* (calculated as the average speed of all the backward (negative) and forward (positive) whisker movements, respectively).

For the first time in a mouse model study (Chapter 3), whisker spread was also quantified and continued to be measured throughout the thesis. Mean angular position and spread are considered the two most informative parameters to assess in whisking (Grant et al. 2009), thus this was an important quantitative metric to supplement the qualitative scoring of spread reduction that is usually measured. *Spread* was scored as the standard deviation of all tracked whisker angular positions. For mean angular position, amplitude, whisker speed and spread, the mean values for right and left whisker measurements were used to give one value per video clip.

Table 2-1 shows the definitions of these whisker variables and predicted PC-DC results based on previous observations. In short, it is expected to see whisker amplitude and asymmetry to all increase following a whisker contact, whereas whisker speeds and spread will decrease following a whisker contact. These behaviours all represent a pattern of the animal to increase the number of whisker contacts (increasing protraction angles and decreasing whisker spread), while ensuring light whisker contacts (increasing asymmetry) over a longer period, by reducing whisker speeds (Grant and Goss, 2022).

Table 2-1 **Whisker parameters measured in this thesis**, pre-contact (PC) and during-contact (DC). Predicted and defined contact-related modifications in whisker movements and related (PC-DC) values are summarised, based on Grant et al. (2018) and Simanaviciute et al. (2022). Arrows correspond to an ↑ - increase or a ↓ - decrease of that whisker variable during-contact, compared to pre-contact. Additionally, to these whisker parameters, locomotion speed was measured in mice (Chapters 3 and 5), but not in rats (Chapter 4).

Whisker parameter	Measurement (defined in PC stage)	Predicted contact-related changes from pre-contact (PC) to during-contact (DC) values	Resulting predicted (PC-DC) values
Locomotion speed (m/s)	Average speed of the centroid of the animal's body.	↓ Decrease locomotion speed during contact	Negative
Amplitude (degrees)	Standard Deviation of the angular positions, multiplied by 2x√2.	↑ Increase amplitude during contact	Negative

Asymmetry (degrees)	Left minus right whisker mean angular position.	↑ Increase asymmetry during contact	Negative
Retraction speed (degrees/s)	Average speed of whiskers moving backwards.	↓ Decrease retraction speed during contact	Positive
Protraction speed (degrees/s)	Average speed of whiskers moving forward.	↓ Decrease protraction speed during contact	Positive
Spread (degrees)	Measures how spread out the whiskers are on average (lower for whiskers bunched up close together, higher for whiskers that are more spread out). The standard deviations of all tracked whisker angular positions, excluding frames that have 0-1 tracked whiskers.	↓ Decrease spread during contact	Positive

2.4 Data analysis

I was always blind to the treatment groups throughout the video collection and analysis process. For object exploration studies, quantitative measures of the pre-contact (PC) whisker variables were first analysed. Then, the changes in whisker measurements during object exploration were analysed by subtracting the during-contact measures from the pre-contact measures (PC-DC), as per Simanaviciute et al. (2022). This technique was shown to robustly reveal contact-related whisker control behaviours during object exploration and have been described in previous studies (Grant et al., 2018, Simanaviciute et al., 2022).

PC-DC was chosen, rather than DC-PC, as it is more intuitive to identify increases in variables during contact as positive, and reductions as negative; in addition, many of the whisking parameters were expected to be higher in PC. For the first time in a mouse model study (Chapter 3), a Linear Mixed-Effects Model was

constructed using the package lme4 (Bates et al., 2015) in R Studio (version 1.1.456) to analyse fixed effects on all PC and PC-DC whisker variables and continued to be used throughout the thesis.

Since the animals were filmed repeatedly exploring an object or the open field, and every subsequent video clip was different, with them acquiring increasingly more information about their environment, each video clip was treated as a within variable, but the degrees of freedom and F-statistics were approximated using a Kenward-Rodger's method (Kenward and Rodger, 1997). This method takes account of uneven and low sample numbers (such as from the 3-month-old 3xTg-AD animals). The degrees of freedom were automatically determined to be anywhere between the number of animals and number of video clips for each particular measurement analysed. Habituation effects within a single session are a potential confound which could be explored by future studies, especially if a different statistical approach is taken; however, this is unlikely to occur in the 10-15 min time frame.

P-values in pairwise comparisons were adjusted with Tukey's method. A significance value of $p < 0.05$ was used throughout. Significant pairwise comparison results are indicated on all figures with one or more asterisks (*). The Kenward-Rodger's approximation is the preferred method of approximating degrees of freedom over Satterthwaite's method (Satterhwaite, 1946; Schaalje et al., 2002), and of reporting p-values over likelihood ratios and Wald t-values (Luke, 2017). It also produces acceptable Type 1 error rates in smaller sample sizes in models fitted with restricted maximum likelihood (Luke, 2017). Satterthwaite's method was also tested to approximate F-tests and degrees of freedom on the quantitative measures. Significant results identified from this method were less conservative than those calculated by the Kenward-Rodger's approach, therefore, increasing the confidence in the chosen statistical reporting. The video data that support the findings of this study will remain indefinitely available from the Manchester Metropolitan University upon request.

CHAPTER 3 ABNORMAL WHISKER MOVEMENTS IN THE 3XTG-AD MOUSE MODEL OF ALZHEIMER'S DISEASE

This chapter has been published as a peer-reviewed journal article and included in the appendix of the thesis:

Simanaviciute, U., Brown, R.E., Wong, A., Fertan, E. and Grant, R.A., 2022. Abnormal whisker movements in the 3xTg-AD mouse model of Alzheimer's disease. Genes, Brain and Behavior, 21(8), p.e12813.

Chapter summary

Alzheimer's disease is the most frequent form of dementia in elderly people. The triple transgenic (3xTg-AD) mouse model of Alzheimer's Disease is important in biomedical research as these mice develop both neuropathological and behavioural phenotypes. However, their behavioural phenotype is variable, with findings depending on the specific task, as well as the age and sex of the mice. Whisker movements reveal motor, sensory and cognitive deficits in mouse models of neurodegenerative disease. Therefore, whisker movements were examined in 3, 12.5 and 17-month-old female 3xTg-AD mice and their B6129S/F2 wildtype controls. Mice were filmed using a high-speed video camera (500 fps) in an open arena during a novel object exploration task. Genotype and age differences were found in mice exploring the arena prior to object contact. Prior to whisker contact, the 3-month-old 3xTg-AD mice had smaller whisker angles compared to the wildtype controls, suggesting an early motor phenotype in these mice. Pre-contact mean angular position at 3 months and whisking amplitude at 17 months of age differed between the 3xTg-AD and wildtype mice. During object contact 3xTg-AD mice did not reduce whisker spread as frequently as the wildtype mice at 12.5 and 17 months, which may suggest sensory or attentional deficits. This study shows that whisker movements are a powerful behavioural measurement tool for capturing behavioural deficits in mouse models that reveal complex phenotypes, such as the 3xTg-AD mouse model.

3.1 Introduction

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disorder, and the most frequent form of dementia in elderly people (Fiest et al., 2016; Lane et al., 2018; Scheltens et al., 2021). Mouse models are essential for improving our understanding of the neural and behavioural changes that occur during AD progression, and to develop novel therapeutic targets (Scearce-Levie et al., 2020, Fertan et al., 2019a). The triple transgenic (3xTg-AD) mouse model is considered to have high validity as these mice develop both Aβ plaques and tau tangles (Oddo et al., 2003), as well as cognitive deficits (Filali et al., 2012; Jankowsky and Zheng 2017). The 3xTg-AD mice have altered performance on sensory tasks involving vision (King et al., 2018), olfaction (Roddick et al., 2016), and touch (Simanaviciute et al., 2020), as well as motor (Stover et al., 2015; Garvock-de Montbrun et al., 2019) and cognitive tasks (Stevens and Brown, 2015; Gür et al., 2019a; Fertan et al., 2019a, b). The 3xTg-AD mice generally perform worse than their wildtype controls in spatial learning and memory tests (Davis et al., 2013; Fertan et al., 2019b). They have a complex motor phenotype and have even shown an enhanced motor phenotype at 6 and 16 months of age (Stover et al., 2015; Garvock-de Montbrun et al., 2019). They show higher frailty measures (Kane et al., 2018) and have a shorter lifespan than their wildtype background strain. Male 3xTg-AD mice also have a shorter lifespan than females (Rae and Brown, 2015), as well as altered immune function and gene expression (Fertan et al., 2019c).

Behavioural studies have shown quite variable outcomes with these mice, especially in motor and cognitive tasks, such as spatial learning and memory (Fertan et al., 2019b; Stover et al., 2015). Age, sex, experimental apparatus and test design all impact the performance of 3xTg-AD mice during behavioural tasks (Kane et al., 2018; Fertan et al., 2019b; Gür et al., 2019b). Therefore, a better understanding of the behavioural manifestations that occur in this model of AD is needed. I suggest that measuring whisker movements in mouse models is an easy and robust way to capture elements of sensory, motor and cognitive deficits in 3xTg-AD mice.

Previous whisker-related studies in the 3xTg-AD mice show that 17-month-old females had smaller whisker angular positions and retraction speeds compared to wildtype controls when moving around their environment without object contacts

(Simanaviciute et al., 2020). However, it is important to measure these changes at different age points to assess when deficits related to AD can be detected and whether the disease progress can be measured. Moreover, whisker movements should be measured during object contact to better understand the sensory and cognitive deficits in whisker movements in this mouse model, considering that open field or pre-contact studies mostly inform about motor deficits. Therefore, the aim of this study is to investigate whisker movements in the 3xTg-AD mouse model at different ages, before and during object exploration. The hypothesis of this work is that AD can be detected at an early age and monitored throughout the different life stages by measuring whisker movements. This study was designed based on the recommendations of Simanaviciute et al. (2020) as shown in Chapter 2 Figure 2-9. To detect sensory, motor, and/or cognitive deficits in the 3xTg-AD mouse model, I tested for all the suggested steps in Figure 2-9. Whisker movements were scored prior to object contact and during object exploration using both qualitative and quantitative measures to detect any deficits in whisking behaviour in the 3xTg-AD mice.

3.2 Materials and methods

Animals

A total of 38 female mice were used in this cross-sectional study: 17 transgenic (3xTg-AD, JAX # 004807) mice (3 at 3 months, 6 at 12.5 months, 8 at 17 months) and 21 wildtype (B6129S/F2 WT, JAX# 101045) mice (8 at 3 months, 7 at 12.5 months, 6 at 17 months). All mice were born in-house at Dalhousie University from breeding pairs purchased from Jackson Laboratory (Bar Harbour, Maine USA). The 3xTg-AD mice were engineered by injecting APPSwe and tauP301L transgenes into single-cell embryos of homozygous PS1M146V knock-in mice. This causes Aβ42 aggregation in the frontal cortex and the hippocampus at around 3 months of age, extracellular plaques in the frontal cortex and the hippocampus at 6 months of age, and hyperphosphorylated tau tangles at 12 months of age (Oddo et al., 2003). This study spans these changes by observing mice from 3 to 17 months of age. Due to increased mortality in male mice by 17 months of age (Rae and Brown, 2015), only female mice were included in this study.

Mice were weaned at 21 days of age, their ears were punched for individual identification, and they were housed in same sex groups of 2–4 in 30 × 18 × 12 cm translucent polycarbonate cages with wire lids and microisolator tops. Cages contained woodchip bedding (Fresh Bed, Shaw Resources, Nova Scotia, Canada) and a 4×7 cm polyvinyl chloride tube for enrichment. They were kept in a climate controlled (22°C ± 2°C) vivarium on a reversed 12-hour light:dark cycle with lights off at 09:45 am. All behavioural testing was completed during the dark (active) portion of the light:dark cycle. Mice had ad libitum access to Purina Laboratory Rodent Chow #5001 (Agribrand Purina, Strathroy, Ont., Canada) and tap water. Mice were treated in accordance with the regulations set forth by the Canadian Council on Animal Care and the experimental protocol was approved by the Dalhousie University Committee on Animal Care and the local ethics committee at Manchester Metropolitan University (reference no. 2019-11562-7372).

Experimental procedures

All experiments were conducted in the animal facility at Dalhousie University, Canada, in the same room with the same experimenter. A Pyrex glass bottle stopper (Figure 3-1A) was placed inside the arena as an object to explore. For all other methods which remain unchanged across the experimental Chapters, please refer to Chapter 2 General methods.

Video analysis: qualitative whisker scores

Whisking by mice was scored on a four-point scale from no whisking (0), to only retractions (1), only protractions (2) or both retractions and protractions (3). To qualitatively assess whisker behaviours and exploratory strategies, all of the video clips that met the above criteria were scored based on a system developed by Grant et al. (2012; 2018a), in which *contact-induced asymmetry*, *spread reduction*, and *head turning asymmetry* were measured (Mitchinson et al., 2011; Towal and Hartmann, 2006). When the mouse was contacting an object with their whiskers, *contact-induced asymmetry* (CIA) was scored on a three-point scale from absent (0), to showing increased contralateral protraction (1), reduced ipsilateral protraction (2) and both increased contralateral protraction and reduced ipsilateral protraction (3). Object-directed whisker *spread reduction* was scored as absent (0) or present (1) when whisker spread decreased following object contact. *Head*

turning asymmetry (HTA) was scored as present (1) or absent (0), during a head turn.

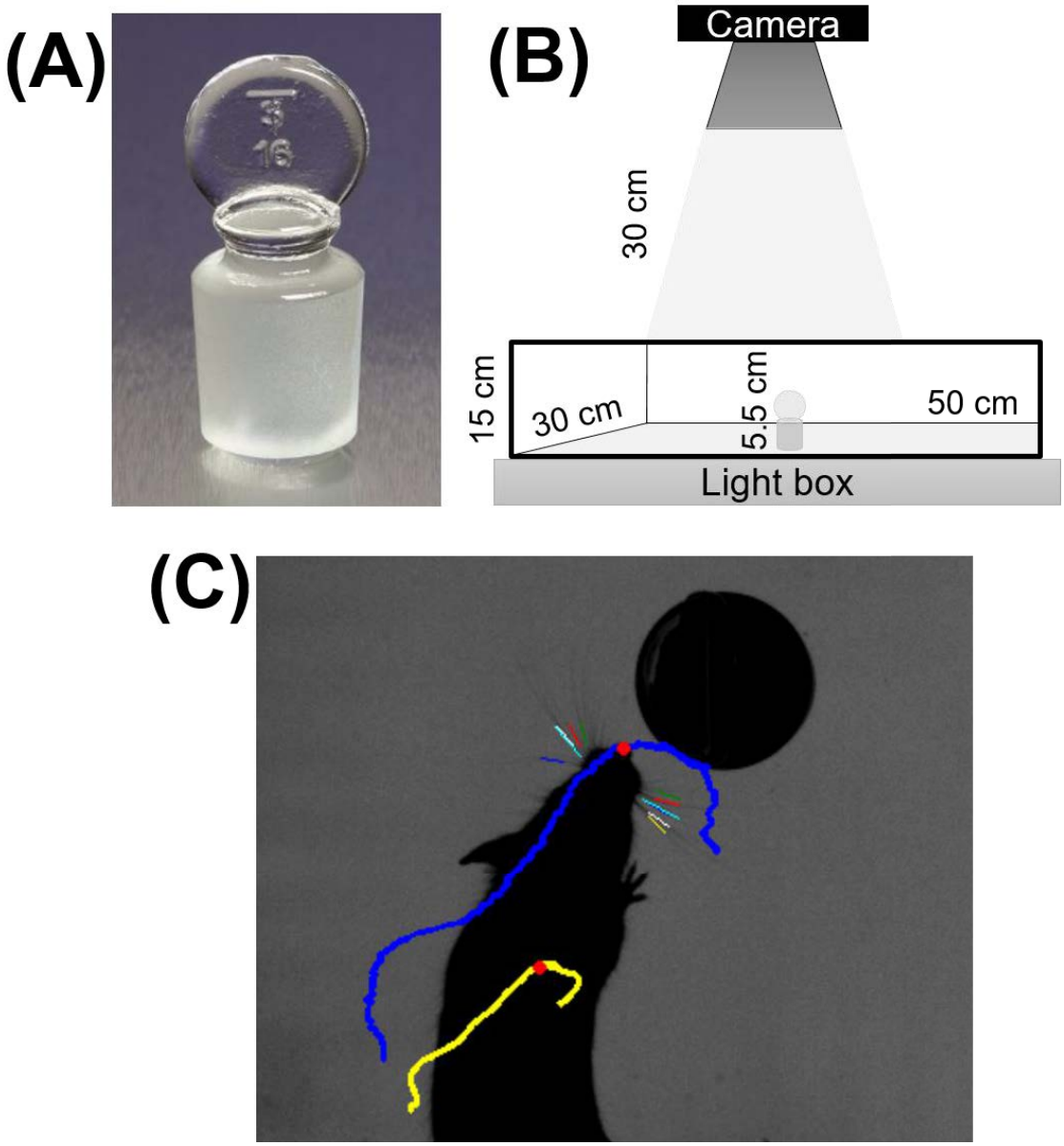


Figure 3-1 **Data collection and video analysis.** Panel A shows the glass bottle stopper object used in the experiments; Panel B illustrates the filming set-up, the object size and location in relation to the Perspex box, and the distance between the arena and high-speed video camera. The field of view in light grey corresponds to the video still in c) showing an example video clip. ARTv2 LocoWhisk software was used to automatically locate the mouse centroid (red point, yellow line), nose tip (red point, blue line) and whiskers (coloured lines), and detects them on a frame-by-frame basis.

Video analysis: quantitative analysis of locomotion and whisker movements

From 1 to 12 video clips per mouse were included in data analysis (Table 3-1), resulting in a total of 183 whole clips, all of which contained both PC and DC sections. PC sections ranged from 100 to 600 frames per clip, whereas DC

sections ranged from 100 to 625 frames. For the first time in a mouse model study, whisker spread was quantified.

Table 3-1 **Number of video clips per mouse included in quantitative analyses.** Genotypes abbreviated: wt – wildtype, tg – 3xTg-AD mice.

Mouse ID	Age (months)	Genotype	No. of clips
3591	3	tg	10
3592	3	tg	3
3600	3	tg	1
3667	3	wt	7
3668	3	wt	2
3670	3	wt	6
3669	3	wt	8
3671	3	wt	2
3677	3	wt	11
3678	3	wt	5
3679	3	wt	12
3351	12.5	wt	4
3352	12.5	wt	3
3353	12.5	wt	4
3354	12.5	wt	5
3355	12.5	wt	1
3356	12.5	wt	7
3365	12.5	wt	7
3369	12.5	tg	4
3370	12.5	tg	1
3371	12.5	tg	1
3379	12.5	tg	2
3380	12.5	tg	1
3381	12.5	tg	3
3160	17	tg	3
3161	17	tg	5
3162	17	tg	6
3166	17	tg	6
3167	17	tg	5
3168	17	tg	5
3193	17	tg	7
3194	17	tg	7
3170	17	wt	8
3174	17	wt	7
3173	17	wt	3
3237	17	wt	5
3180	17	wt	5
3181	17	wt	1
38 total			183 total

Statistical analyses

For all qualitative and quantitative whisker measurements, each variable was compared between wildtype and 3xTg-AD mouse, at each age (3, 12.5 and 17 months). Qualitative scores of whisking behaviours were analysed using the Kruskal-Wallis test with Dunn's post-hoc tests using GraphPad Prism 8 software, as these were on ordinal scales and not normally distributed.

For quantitative measures, for the first time in a mouse model study, a Linear Mixed-Effects Model was constructed to analyse the effect of age and genotype on all PC and PC-DC whisker variables. The model computed *F* tests on the fixed effects of age and genotype and provided p-values using a type III ANOVA, as well as interaction effects (although all the interaction effects were not significant and will not be referred to further in the main text, though, see Table 3-3 and Table 3-4 for more detail).

3.3 Results

Qualitative whisker behaviour

The whisking scores from the qualitative measures show that, while all wildtype mice whisked, with median score 3, the 3xTg-AD mice had lower scores with medians of 2 - 3 ($H(5, 183) = 39.9$, $p < 0.001$; Figure 3-2A). At 12.5 months ($p = 0.008$) and 17 months ($p < 0.001$) of age the 3xTg-AD mice had significantly reduced whisking scores compared to the age-matched wildtypes, revealing more whisking movements which were only protractions in the 3xTg-AD mice, rather than the protractions and retractions associated with whisking in the wildtype mice. The whisking scores of the 17-month-old 3xTg-AD mice were also significantly lower than those of the 3-month-old 3xTg-AD mice ($p = 0.020$). There were no significant differences in HTA scores between 3xTg-AD and wildtype mice ($H(5, 101) = 6.74$, $p = 0.241$, Figure 3-2B). During object exploration there were significant differences in spread reduction ($H(5, 183) = 20.6$, $p < 0.001$) and CIA ($H(5, 183) = 26.4$, $p < 0.001$) between 3xTg-AD and wildtype mice. The 12.5-month-old 3xTg-AD mice had significantly lower whisker spread reduction values than their wildtype controls ($p = 0.008$), and these were also lower than the values

for 3-month (p = 0.003) and 17-month (p = 0.002) 3xTg-AD mice (Figure 3-2C). The CIA scores of the 3-month-old wildtype mice were significantly higher than the age-matched 3xTg-AD mice (p = 0.008) and the 12.5-month wildtype mice (p < 0.001, Figure 3-2D). Detailed statistical information for every comparison in qualitative analyses can be found in Table 3-2.

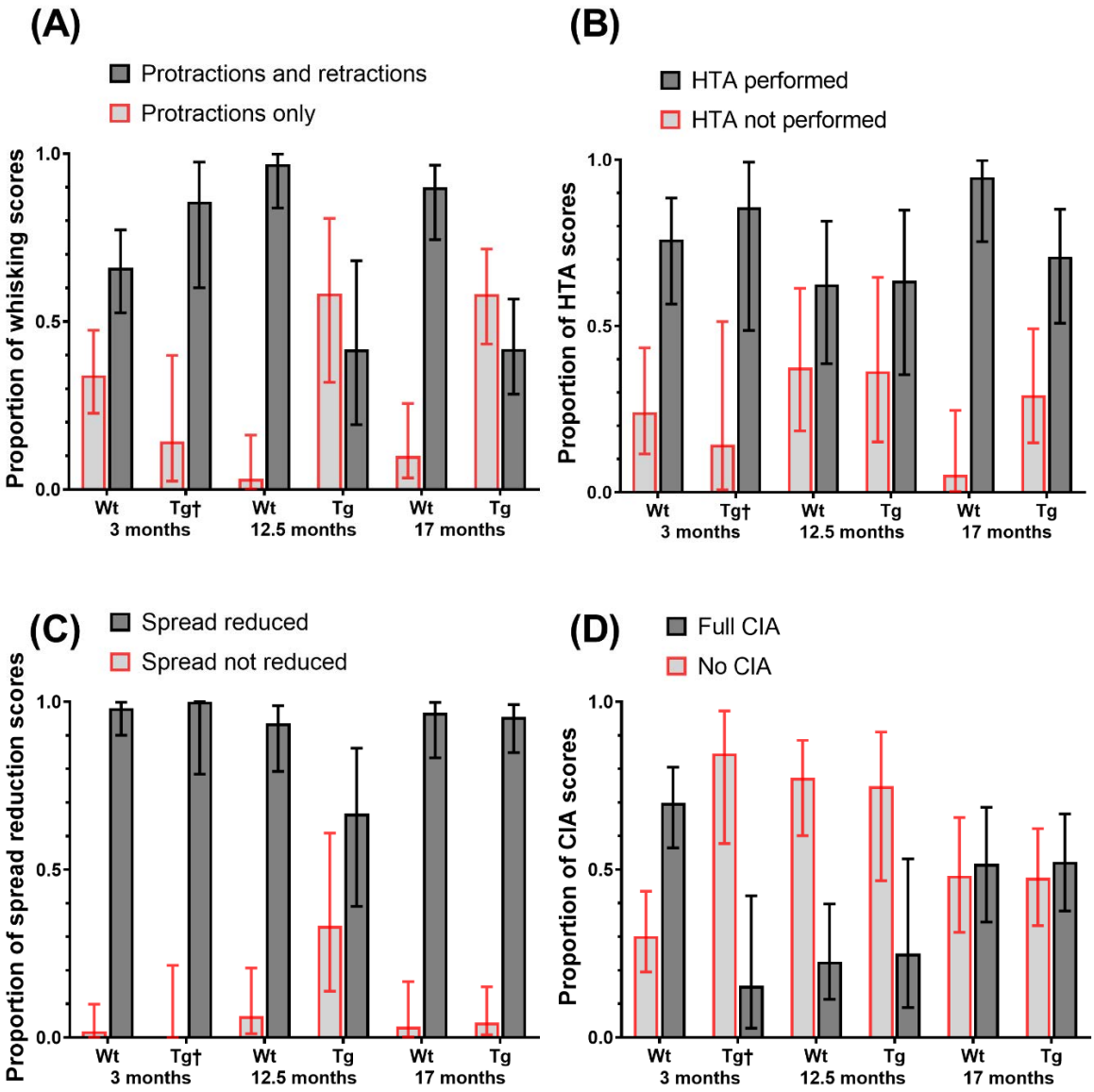


Figure 3-2 **Qualitative whisker behaviour scores for (A) whisking, (B) head turning asymmetry (HTA), (C) spread reduction, (D) contact-induced asymmetry (CIA).** The bars indicate the proportion of clips where the behaviour occurred or did not occur, with confidence intervals. † indicates n=3 mice.

Table 3-2 **Summary statistics for qualitative data.** Kruskal-Wallis test and Dunn's post-hoc. Asterisks mark significant values where $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

	Main effect $df_1 = 5$, df_2, H, p	3mon Wt vs 3mon Tg Z, p	12.5mon Wt vs 12.5mon Tg Z, p	17mon Wt vs 17mon Tg Z, p	3mon Wt vs 12.5mon Wt Z, p	3mon Wt vs 17mon Wt Z, p	12.5mon Wt vs 17mon Wt Z, p	3mon Tg vs 12.5mon Tg Z, p	3mon Tg vs 17mon Tg Z, p	12.5mon Tg vs 17mon Tg Z, p
Whisking scores	183 39.90 <0.001 ***	1.40 1.000	3.47 0.008 **	4.51 <0.001 ***	2.91 0.054	2.25 0.370	0.57 1.000	2.40 0.247	3.18 0.022 *	0.010 1.000
CIA	183 26.40 <0.001 ***	3.47 0.008 **	0.14 1.000	0.20 1.000	4.21 <0.001 ***	1.75 1.000	2.16 0.467	0.36 1.000	2.26 0.360	1.69 1.000
Spread reduction	183 20.60 <0.001 ***	0.28 1.000	3.48 0.008 **	0.23 1.000	0.89 1.000	0.28 1.000	0.54 1.000	3.73 0.003 **	0.65 1.000	3.89 0.002 **
HTA	101 6.74 0.241	0.53 1.000	0.17 1.000	1.80 1.000	0.81 1.000	1.42 1.000	2.00 0.683	1.19 1.000	0.80 1.000	0.62 1.000

Pre-contact (PC) quantitative whisker and locomotion movements

For pre-contact whisker amplitude, there were significant main effects of both genotype (F (1, 29.36) = 12.43, $p = 0.001$) and age (F (2, 27.08) = 4.06, $p = 0.029$). Specifically, pre-contact whisker amplitude was lower in 3xTg-AD mice than in the age-matched wildtype mice (Figure 3-3A). Pairwise tests show that these differences were significant in 17-month-old mice ($p = 0.013$). These differences can also be seen in the pre-contact whisker traces in Figure 3-4. Furthermore, there was a difference in pre-contact whisker amplitude between 3 and 17-month wildtype mice ($p = 0.042$) as whisker amplitude increased with age.

For the pre-contact whisker angular position, there were significant main effects of genotype (F (1, 32.82) = 20.38, $p < 0.001$) and age (F (2, 32.66) = 6.96, $p = 0.003$). The pre-contact whisker angular position was consistently lower in the 3xTg-AD mice compared to the wildtype mice (Figure 3-3B), especially at 3 months of age ($p = 0.040$). These results are supported by the video stills (Figure 3-5) and the whisker traces (Figure 3-4), where pre-contact mean whisker angles were lower in the 3xTg-AD mice than the wildtype mice. Wildtype mice at 3

months of age also had larger pre-contact mean angular positions than wildtype mice at 12.5 months (p = 0.038) and 17 months of age (p = 0.006).

In the pre-contact whisker spread, there were significant main effects of genotype (F (1, 32.83) = 10.62, p = 0.003) and age (F 2, (32.67) =5.61, p = 0.008). However, pairwise tests did not reveal any significant differences (Figure 3-3C). There were no significant differences in pre-contact whisker movements in locomotion speed, asymmetry, retraction speed and protraction speed (Figure 3-6). Detailed statistical information for every comparison in PC quantitative analyses can be found in Table 3-3.

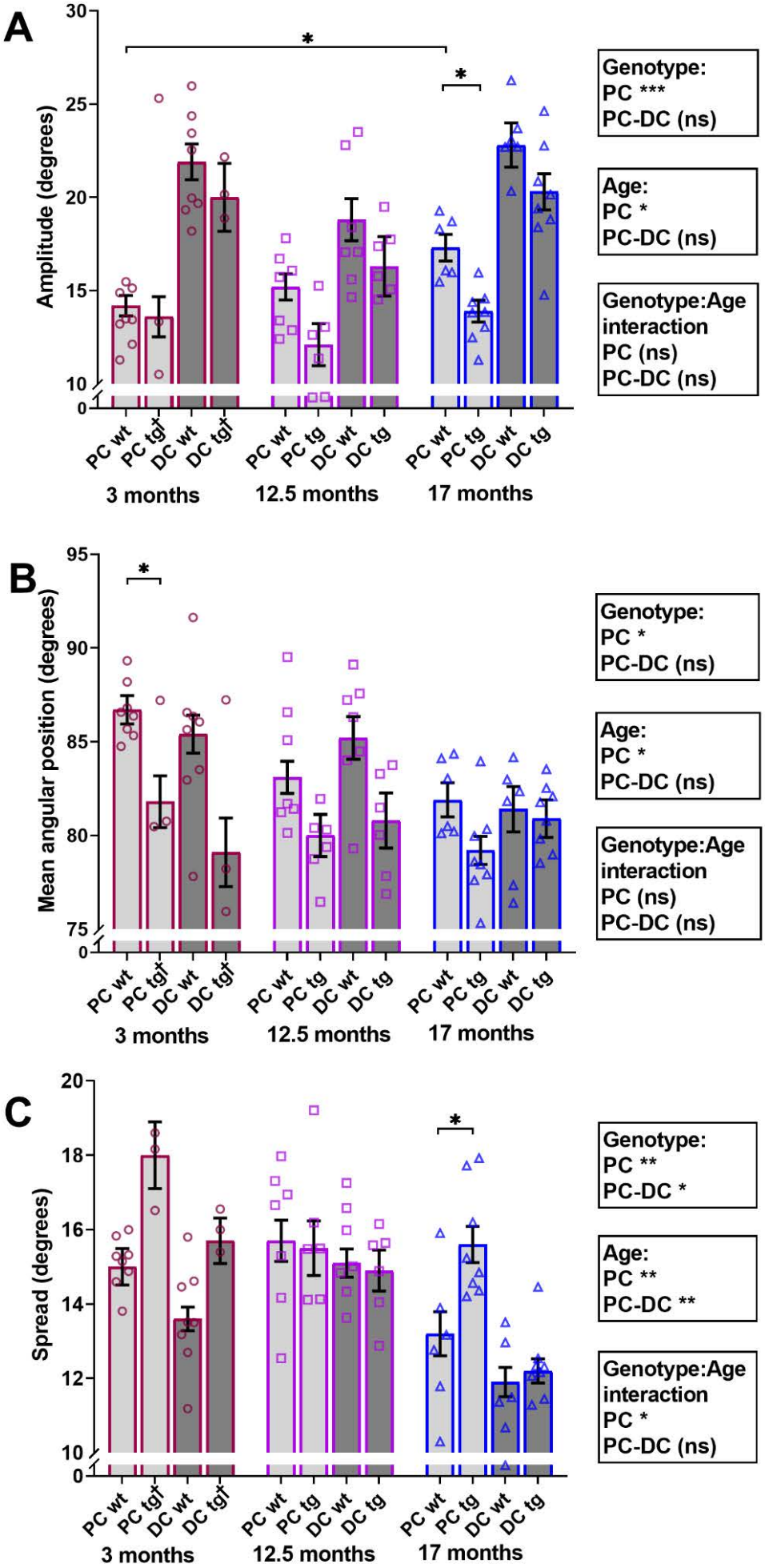

A
Amplitude (degrees)
30
25
20
15
10
0
*
*
PC wt
PC tg†
DC wt
DC tg†
PC wt
PC tg
DC wt
DC tg
PC wt
PC tg
DC wt
DC tg
3 months
12.5 months
17 months
Genotype:
PC ***
PC-DC (ns)
Age:
PC *
PC-DC (ns)
Genotype:Age
interaction
PC (ns)
PC-DC (ns)
B
Mean angular position (degrees)
95
90
85
80
75
0
*
PC wt
PC tg†
DC wt
DC tg†
PC wt
PC tg
DC wt
DC tg
PC wt
PC tg
DC wt
DC tg
3 months
12.5 months
17 months
Genotype:
PC *
PC-DC (ns)
Age:
PC *
PC-DC (ns)
Genotype:Age
interaction
PC (ns)
PC-DC (ns)
C
Spread (degrees)
20
18
16
14
12
10
0
*
PC wt
PC tg†
DC wt
DC tg†
PC wt
PC tg
DC wt
DC tg
PC wt
PC tg
DC wt
DC tg
3 months
12.5 months
17 months
Genotype:
PC **
PC-DC *
Age:
PC **
PC-DC **
Genotype:Age
interaction
PC *
PC-DC (ns)

Figure 3-3 **Mean angular position, amplitude and spread are affected by genotype and age.** All significant differences are between 3xTg-AD and wildtype mice, unless otherwise specified. Panel A: Significant age and genotype effects were found in pre-contact mean angular whisker positions. Pairwise comparisons showed a significant difference in the 3-month age group. Panel B: Significant age and genotype effects were found in pre-contact whisker amplitudes. Pairwise comparisons showed a significant difference in the 17-month age group between 3xTg-AD and wildtype mice, as well as between 3 and 17-month wildtype mice. Panel C: Significant age and genotype effects were found in pre-contact whisker spread. Age and genotype effects were found in contact-related (PC-DC) spread. Pairwise comparisons showed a significant difference in the 17-month age group in (PC-DC) spread as well as between 12.5 and 17-month wildtype mice. The bars indicate the mean values from all the clips (degrees of freedom calculated from a linear mixed-effect model), with standard error bars. Asterisks mark significant values where $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***. Data points show mean values for individual mice, indicated by circles for 3-month mice, squares for 12.5-month mice, triangles for 17-month mice. PC = pre-contact, DC = during contact, PC-DC = contact related behaviours. † indicates n=3 mice.

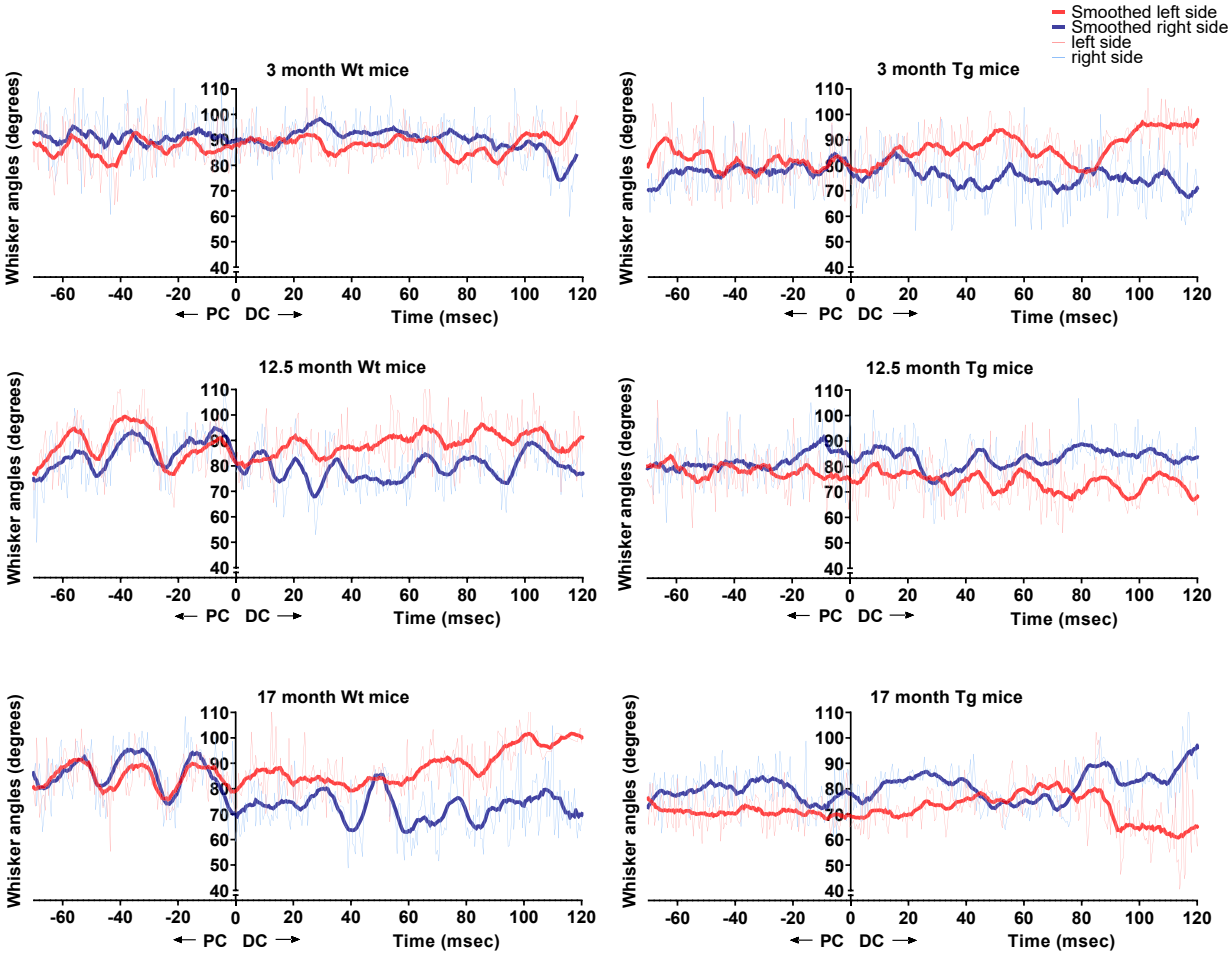


Figure 3-4 **Example whisker angle traces of wildtype and 3xTg-AD mice at each age.** Raw data points are shown in fine lines, and smoothed data (2nd order, 15 neighbours) are presented in thicker lines. Red colour traces are from the whiskers on the left side, and blue from the right side. 0 msec is the point of contact on the x-axis; therefore, left from the Y-axis is PC and right from the Y-axis is DC.

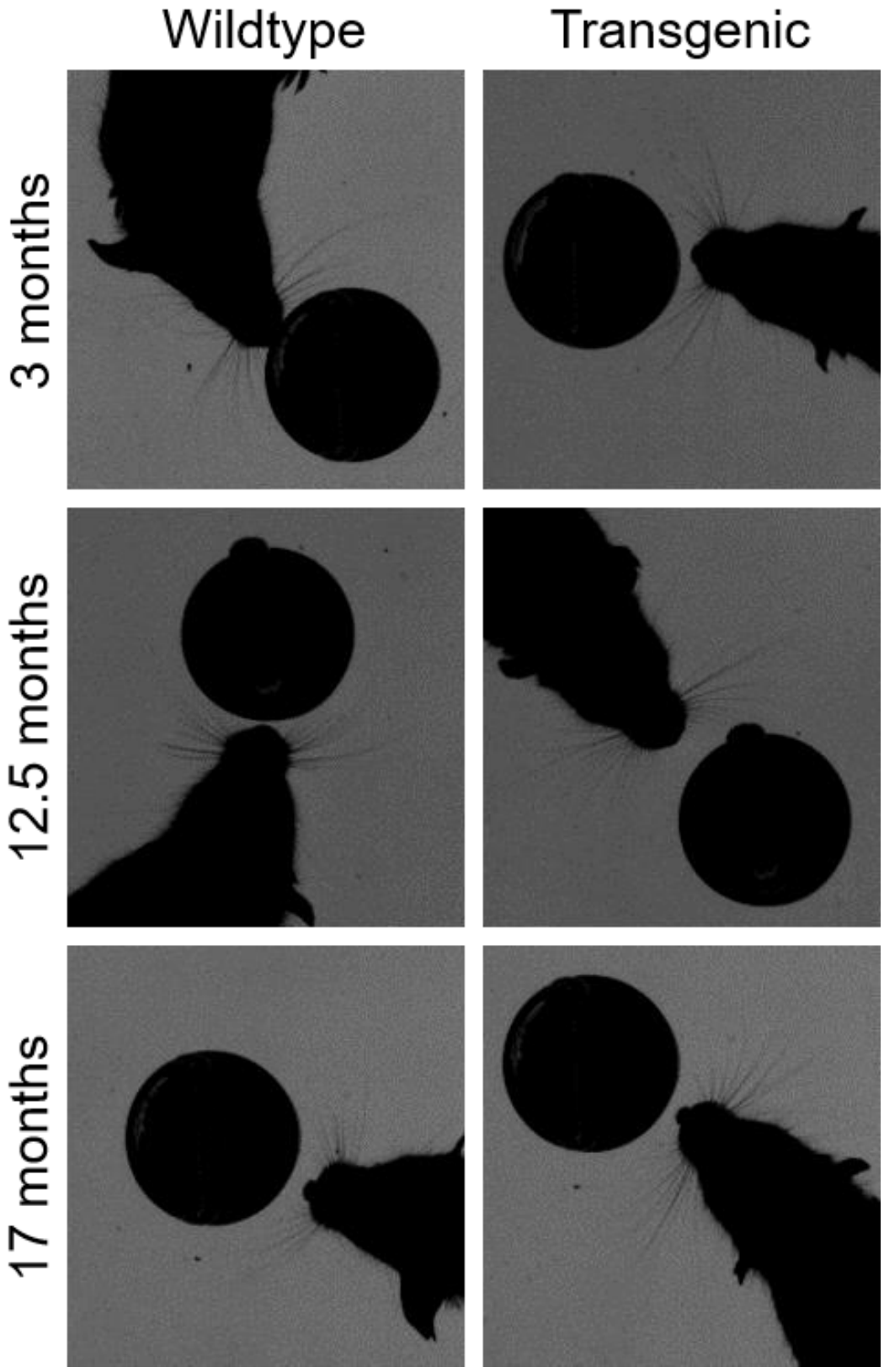


Figure 3-5 **Whiskers are more spread out in 3xTg-AD mice during object contact.** Video stills of representative mice are shown contacting the object, where whiskers are at maximum protraction. Whiskers of the wildtype mouse are positioned more forward towards the object and less spread out, compared to the 3xTg-AD mouse, especially at 3 and 17 months.

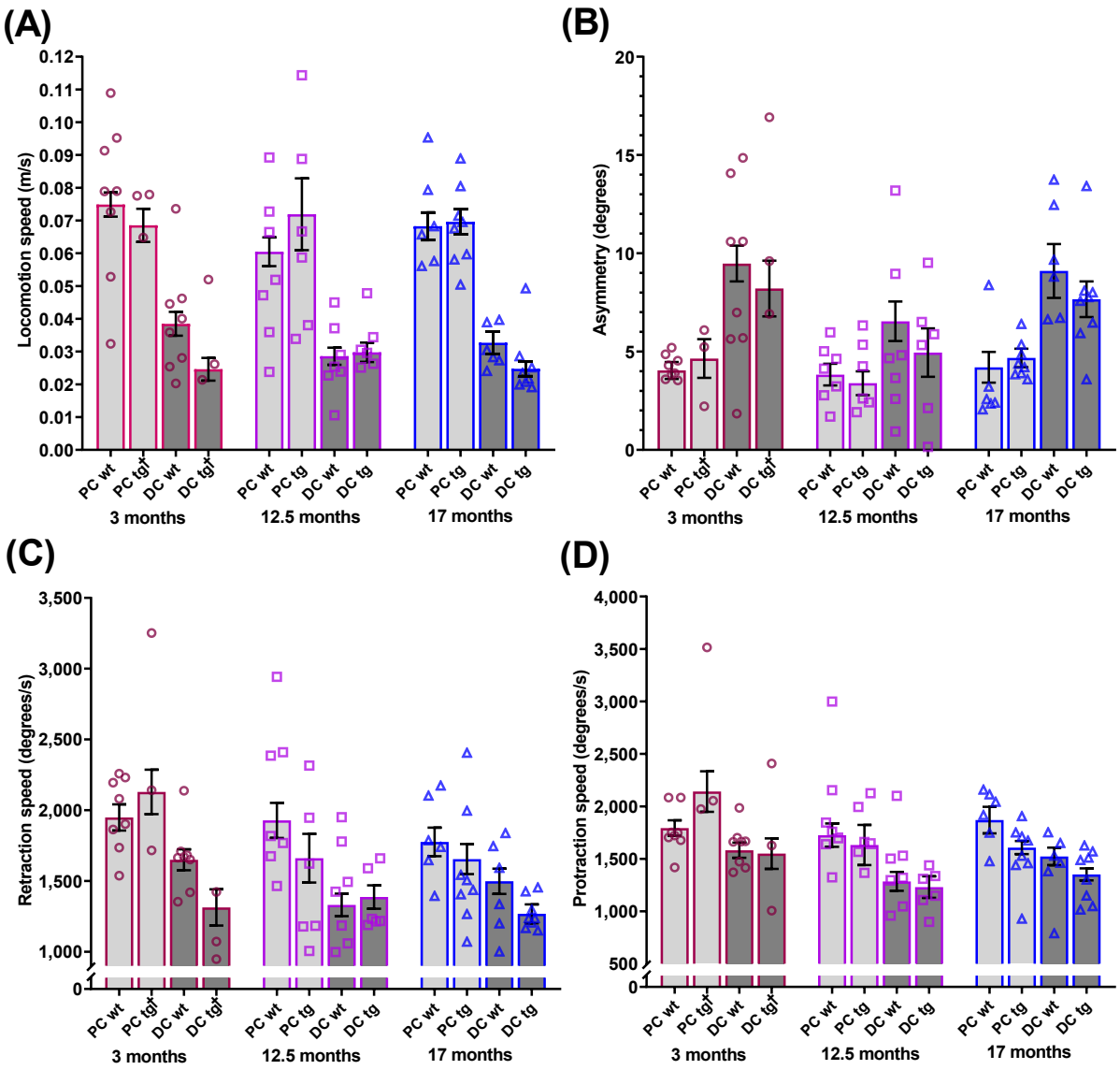


Figure 3-6 **Other locomotion and whisker variables tested: (A) locomotion speed, (B) asymmetry, (C) retraction speed and (D) protraction speed.** There were no significant effect of age or genotype on any of these variables. The bars indicate the mean values from all the clips (degrees of freedom calculated from a linear mixed-effect model), with standard error bars. Data points show mean values for individual mice, indicated by circles for 3-month mice, squares for 12.5-month mice, triangles for 17-month mice. PC = pre-contact, DC = during contact, PC-DC = contact related behaviours. † indicates n=3 mice.

Table 3-3 **Summary statistics for quantitative pre-contact data.** Linear mixed effect model and pairwise comparisons with Tukey's adjustment. Asterisks mark significant values where $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

PC	Genotype effect $df_1 = 1$, df_2, F, p	Age effect $df_1 = 2$, df_2, F, p	Genotype: Age interaction $df_1 = 2$, df_2, F, p	Post-hoc: **Tg vs Wt 3mon** df, t-ratio, p	Post-hoc: **Tg vs Wt 12.5mon** df, t-ratio, p	Post-hoc: **Tg vs Wt 17mon** df, t-ratio, p
Locomotion speed (m/s)	33.09 0.079 0.780	32.99 0.88 0.426	32.99 0.36 0.697	30.40 -0.21 1.000	42.60 0.86 0.954	27.80 -0.16 1.000
Amplitude (degrees)	29.36 12.43 0.001 **	27.08 4.06 0.029 *	27.08 1.57 0.227	5.10 -0.54 0.994	66.30 -2.60 0.113	25.70 -3.27 0.033 *
Mean angular position (degrees)	32.82 20.38 < 0.001 ***	32.66 6.96 0.003 **	32.66 0.61 0.549	28.90 -3.15 0.040 *	44.90 -2.21 0.255	27.20 -2.42 0.184
Asymmetry (degrees)	29.65 0.55 0.463	27.75 0.30 0.743	27.75 0.28 0.754	16.30 0.48 0.996	63.70 -0.12 1.000	25.60 1.13 0.866
Retraction speed (degrees/s)	31.01 0.74 0.398	30.34 2.37 0.110	30.34 1.01 0.377	21.70 0.66 0.985	54.70 -1.18 0.845	25.80 -1.15 0.854
Protraction speed (degrees/s)	29.94 0.011 0.916	28.34 2.54 0.097	28.34 2.92 0.071	17.30 1.72 0.538	61.60 -0.59 0.992	25.60 -1.69 0.551

Spread (degrees)	32.83 10.62 0.003 **	32.67 5.61 0.008 **	32.67 3.22 0.053	28.90 2.91 0.069	44.9 -0.20 1.000	27.20 3.05 0.051

Contact-related (PC-DC) quantitative whisker and locomotor movements

Both wildtype and 3xTg-AD mice showed robust changes in whisker movements in response to object contact at all ages as indicated by a reduction in locomotion speed (Figure 3-6A), retraction and protraction speeds (Figure 3-6C and D), and an increase in whisker asymmetry (Figure 3-6B) and amplitude (Figure 3-3A) following an object contact (PC-DC). The whisker traces (Figure 3-4) show this increase in asymmetry as the left (red) and right (blue) traces separate following object contact in all examples. Since these behaviours were robust in all mice, there were no significant effects of genotype or age in the contact-related (PC-DC) variables of whisker amplitude (Figure 3-3A), whisker angular position (Figure 3-3B), locomotion speed, whisker asymmetry, retraction speed and protraction speed (all Ps>0.05, Figure 3-6A-D). However, in (PC-DC) whisker spread, there were significant main effects of genotype ($F(1, 29.79) = 4.60$, $p = 0.040$) and age ($F(2, 28.04) = 6.79$, $p = 0.004$) as (PC-DC) whisker spread was significantly higher in the 3xTg-AD mice than the wildtype mice at 17 months of age ($p = 0.041$; Figure 3-3C and Figure 3-5). There was also a significant difference between 12.5-month and 17-month transgenic mice, with the 17-month transgenic mice reducing their spread more upon contact ($p = 0.007$) than the 12.5-month mice. Detailed statistical information for every comparison in PC-DC quantitative analyses can be found in Table 3-4.

Table 3-4 **Summary statistics for quantitative contact-related (PC-DC) data.** Linear mixed effect model and pairwise comparisons with Tukey's adjustment. Asterisks mark significant values where $p \leq 0.05$ = *, $p \leq 0.01$ = **.

PC-DC	Genotype effect $df_1 = 1$, df_2, F, p	Age effect $df_1 = 2$, df_2, F, p	Genotype: Age interaction $df_1 = 2$, df_2, F, p	Post-hoc: **Tg vs Wt 3mon** df, t-ratio, p	Post-hoc: **Tg vs Wt 12.5mon** df, t-ratio, p	Post-hoc: **Tg vs Wt 17mon** df, t-ratio, p
Locomotion speed (m/s)	32.36 1.07 0.309	32.10 0.25 0.777	32.10 0.020 0.981	26.70 0.49 0.996	47.80 0.73 0.977	26.60 0.59 0.991
Amplitude (degrees)	30.19 0.014 0.907	28.82 2.49 0.101	28.82 0.57 0.572	18.30 0.84 0.956	60.00 -0.16 1.000	25.60 -0.64 0.986
Mean angular position (degrees)	32.61 0.028 0.869	32.41 3.12 0.058	32.41 1.54 0.230	27.90 0.75 0.973	46.30 0.66 0.986	26.90 -1.46 0.691
Asymmetry (degrees)	32.04 0.66 0.421	31.68 1.22 0.308	31.68 0.21 0.815	25.40 0.078 1.000	49.60 0.38 0.999	26.40 1.13 0.866
Retraction speed (degrees/s)	29.97 0.49 0.489	28.40 0.97 0.391	28.40 2.46 0.104	17.40 2.02 0.369	61.40 -1.12 0.871	25.60 0.37 0.999
Protraction speed (degrees/s)	29.22 0.50 0.485	26.71 0.48 0.624	26.71 1.68 0.206	14.50 1.80 0.493	67.70 -0.17 1.000	25.70 -0.57 0.992

Spread (degrees)	29.79 4.60 0.040 *	28.04 6.79 0.004 **	28.04 2.00 0.155	16.80 1.26 0.800	62.70 -0.16 1.000	25.60 3.16 0.041 *

3.4 Discussion

Whisker tracking is not directly translatable to humans and one particular type of AD-like mutation in mice is not a full representation of that disease in humans. Thus, no comparisons to human symptoms will be made in this discussion. As hypothesized, the 3xTg-AD mice differed from age-matched wildtype mice in their whisker movements, both prior to and during object exploration. Specifically, I observed significant genotype differences in pre-contact whisking scores, mean angular position and whisking amplitude, as well as during-contact whisker spread, spread reduction scores and contact-induced asymmetry scores. I suggest that these observations may correspond to a whisker motor phenotype in 3xTg-AD mice from 3 months of age and a sensory or attentional deficit, associated with contact-related whisker movements, at 12.5 and 17 months of age.

Pre-contact movements

Prior to any object contact, the whisking movements of the 3xTg-AD mice differed from the wildtype mice. The qualitative whisking scores showed that 12.5 and 17-month 3xTg-AD mice did not always make full retraction movements during whisking compared to the wildtypes (Figure 3-2A). Whisker tracking revealed that mean angular positions of 3xTg-AD mice were consistently lower than the wildtype mice, and significantly so at 3 months (Figure 3-3B). Moreover, pre-contact amplitude was significantly lower in 17-month-old 3xTg-AD mice compared to the wildtypes. These findings suggest the presence of a motor phenotype in 3xTg-AD mice, from perhaps as early as 3 months of age. However, the exact age of this phenotype is unclear from this data and is likely to depend on the exact measure, since it varies between the measures of whisking, whisker angle and amplitude.

The 3xTg-AD mice are known for complex age-related motor abnormalities. The 3xTg-AD mice often perform better than non-transgenic mice in rotarod tasks (Blanchard et al., 2010 at 6-7 months; Filali et al., 2012 at 12-14 months; Chen et al., 2014 at 6 months; Stover et al., 2015 at 6 months; Garvock-de Montbrun et al., 2019 at 16 months) and have longer stride lengths during locomotion (Stover et al., 2015). However, other studies have shown that the stride length (Setogawa et al., 2014; Filali et al., 2012), walking speed (Setogawa et al., 2014; Stover et al., 2015) and rotarod performance (Sterniczuk et al., 2010; Setogawa et al., 2014) can also be unaffected in 3xTg-AD mice. Indeed, locomotion speed was not significantly affected in these mice. However, it is worth noticing that locomotion speed was only measured in several frames as the mouse approached an object, therefore, it is not comparable to the gait analysis or rotarod and balance beam set-ups used by other studies. Some studies have even shown a reduced motor phenotype in 3xTg-AD mice. For instance, Garvock-de Montbrun et al. (2019) showed that, despite the enhanced rotarod performance, 3xTg-AD mice at 16 months of age display a reduction in walking distance and speed compared to the wildtype mice in a balance beam task, suggesting an age-related decline in motor performance. Orta-Salazar et al. (2019) also found a reduction in locomotion distance and time in 11-month 3xTg-AD mice in an open field test. Overall, no evidence of an enhanced motor phenotype in the 3xTg-AD mice was observed. In fact, these results are more in favour of a reduced motor phenotype, starting from reduced whisker angles at 3 months, and then seeing changes in whisking capacity at 12.5 and 17 months, later also showing up as reduced whisker amplitude at 17 months. One issue in the analysis of motor phenotypes in the 3xTg-AD mice is the background strain used. Background strains can have a significant effect on behavioural phenotypes (Fertan et al., 2021) and the 3xTg-AD mice are available from the JAX Labs on three different backgrounds: B6;129 (Stock No. 004807), 129S4 (Stock No. 0319881), and C57BL/6J (Stock No. 033930). Recent research (Castillo-Mariqueo and Giménez-Llort, 2021) suggests that the motor phenotype of the 3xTg-AD mice on the C57Bl6 background differs from that of the mice on the B6129 background that was used here.

I observed variation in whisker movements between wildtype mice of different ages. Specifically, pre-contact whisker amplitude was significantly higher in 17-month wildtype mice compared to 3-month wildtype mice, and pre-contact mean angular position was significantly higher in 3-month wildtype mice compared to older mice. Very young mice (10-13-days-old) also have smaller whisker amplitudes than weaned (21-days-old) mice (Grant et al., 2012). Therefore, there might be a tendency for pre-contact whisker amplitude to increase with age in wildtype mice. Although studies of age-related changes in whisker movements are few, Garland et al. (2018) show a visible amplitude increase in older wildtype mice when testing Q175, Hdh Q150 and Hdh Q250 mouse models of Parkinson's disease (all mice tested at 10, 20 and 90 weeks, Hdh Q150 and Hdh Q250 mice also tested at 55 weeks; amplitude increasing at every age). They also show decreasing mean angular position in wildtype mice when testing the R6/2 CAG250 mice (decreasing from 8 to 10 weeks and from 12 to 18 weeks). However, these age-related changes were not statistically evaluated in their work. Investigating the changes in whisker movements over an animal's lifecycle would be a useful addition to this work.

Data from 17-month-old mice analysed by Simanaviciute et al. (2020) agreed with this study, as they found that 17-month-old female 3xTg-AD mice had lower whisker angular positions than wildtype mice. However, they also found that retraction speed was significantly lower in the 3xTg-AD mice. While retraction speed was consistently lower in 3xTg-AD mice compared to the wildtype mice (Figure 3-6C) in this study, this difference was not statistically significant. Simanaviciute et al. (2020) used per-clip measures for statistical analyses, whereas here a stricter linear mixed effect model was used. In statistical analyses, treating every trial as an independent data point can lead to pseudo-repetition (Lazic et al., 2018) and inflate the power of the statistical test. Therefore, per-trial, or, in this case, per-clip measures should not be used as independent data points, despite this often occurring in animal studies, especially where the sample size drops due to unforeseen experimental circumstances or data quality issues. In this case, it is recommended to use a mixed-effect model that automatically

determines degrees of freedom for the dataset instead of using standard parametric and non-parametric tests (Boisgontier and Cheval, 2016; Judd et al., 2012), which has informed the approach used here.

Contact-related movements

The 3xTg-AD and wildtype mice at all ages made robust object contact-related whisker movements, as indicated by a decrease in whisker speeds, spread, and increased amplitude and asymmetry following whisker contact (Figure 3-3B and 4C for amplitude and spread; Figure 3-6 for all other parameters). Contact-related spread was affected in the 3xTg-AD mice, compared to the wildtype control mice. In the qualitative scoring, 12.5-month 3xTg-AD mice reduced whisker spread following contact less often than the controls. In the quantitative tracking, contact-related whisker spread was significantly higher in the 3xTg-AD mice than wildtypes at 17 months. 17-month 3xTg-AD mice also reduced their whisker spread following contact more than 12.5-month 3xTg-AD mice. It is unknown exactly what the sensory implications are of reducing whisker spread following contact, although it seems to play a role in increasing the number of whiskers contacting an object (Grant et al., 2009). Why there is a difference in age in the spread reduction upon contact is not clear and demonstrates the need for more research in this field.

While some of these contact-related changes tend to be robust across animals (Simanaviciute et al., 2020), some are still relatively variable and do not occur on every object contact. For example, 3-month-old wildtype mice show CIA significantly more often than other wildtype or 3xTg-AD mice (Figure 3-2D), and HTA seems to be quite variable (Figure 3-2B). The reason for this is unknown, although it is likely due to variation in behaviour and motivation between individuals. Spread reduction, HTA and CIA have all been associated with orienting of the whiskers towards a region in space or an object, and hence with the animal's attention (Mitchinson and Prescott, 2013). Contact-related whisker movement deficits observed in whisker spread and spread reduction could, therefore, imply an attentional deficit in 3xTg-AD mice. Attentional deficits have previously been documented in these mice in a visual task (Romberg et al., 2011),

although in any sensory task it is challenging to separate attentional and sensory deficits (Romberg et al., 2011). Overall, the results suggest that contact-related sensory or attentional whisker movement deficits are likely to be present in 12.5 and 17-month-old 3xTg-AD mice.

Placing whisker movements into findings from other tests

I further compare these findings with those of other studies involving behavioural and cognitive tasks in Table 3-5. Overall, in this study, and those of Stevens and Brown (2015) and Fertan et al. (2019), there is an early behavioural phenotype at 2-4 months old. This age group shows the most deficits in working memory and spatial learning (Stevens and Brown 2015; Fertan et al. 2019), despite being at the early stage of Alzheimer's disease. The current study shows contact-related whisker movement differences at this age too – especially in contact-induced asymmetry scores and asymmetry, which may be associated with attentional or cognitive disturbances. An early motor phenotype was also described, with pre-contact whisking amplitude significantly affected in these young mice. The 6-month group, which was not tested here, did not show any differences in the previous studies (Stevens and Brown 2015; Fertan et al. 2019b); however, they observed some significant differences in working memory and spatial learning in the 12–13-month-old mice (Table 3-5). Differences were also observed in contact-related spread reduction and in pre-contact whisking scores at this age, perhaps indicating both motor and cognitive deficits at this age. Surprisingly, deficits observed in 12–13-month-old mice were not maintained in older animals at 15 months in the studies by Stevens and Brown (2015). The 15-month-old animals were not tested here; however, at 17 months, mice showed differences in both contact related and pre-contact measures, with whisking scores being maintained from the 12-13-month-old group. This suggests that in later stages of the disease, whisker movement measurements might be a better test to adopt than other, more standard behavioural tasks.

Table 3-5 **The comparison between the findings in whisker movements of this study and other behavioural and cognitive studies conducted in the same laboratory.** † - 3-month 3xTg-AD group n = 3, number of clips is 14. Asterisk (*) indicates assessment of working memory and spatial learning. Dash (-) – not applicable which means it was not tested, as opposed to n.s. which indicate that the measure was tested but was not significantly different from controls. A tick (✓) corresponds to significant differences in the results.

Measure where 3xTg **female** mice show differences from controls	2-4mon	6-7mon	12-13mon	15mon	17mon	Reference
More working memory errors in radial maze	✓	n.s	✓	n.s	-	Stevens and Brown 2015
More reference memory errors in radial maze	✓	n.s	n.s	n.s	-	Stevens and Brown 2015
Less correct entries in the first four arms in radial maze	✓	n.s	n.s	n.s	-	Stevens and Brown 2015
More total arm entries in radial maze	✓	n.s	n.s	n.s	-	Stevens and Brown 2015
More errors in hard Hebb-Williams maze*	n.s	n.s	✓	-	-	Fertan et al., 2019c
Shorter latencies in easy Hebbs-Williams mazes*	✓	n.s	✓	-	-	Fertan et al., 2019c
Shorter latencies in intermediate Hebbs-Williams mazes*	✓	n.s	n.s	-	-	Fertan et al., 2019c
Reduced whisking scores	n.s	-	✓	-	✓	Current study results
Lower spread reduction scores	n.s	-	✓	-	n.s	Current study results
Lower contact-induced asymmetry scores	✓†	-	n.s	-	n.s	Current study results
Lower pre-contact whisker amplitude	n.s	-	n.s	-	✓	Current study results
Lower pre-contact whisker angular position	✓†	-	n.s	-	n.s	Current study results
Higher during-contact whisker spread	n.s	-	n.s	-	✓	Current study results

Limitations and future recommendations

After the data selection process using previously validated criteria, only three mice could be included in the 3-month 3xTg-AD group. This sample size is low; however, the data was kept with additional indication for a low sample size in figures and figure captions. Moreover, the statistical method selected is appropriate to make the most of the uneven sample sizes. The difficulty of including more clips from this group might indicate that the 3-month 3xTg-AD mice behave differently from the other groups, since their clips did not often fit the selection criteria, while a lot more clips were able to be included from their control group. This could mean that the data collection method needs to be refined to focus on collecting more clips from the young mice, given that in the previous studies from this laboratory (Stevens and Brown 2015; Fertan et al., 2019c) as well as this study, the young female mice seem to be affected the most.

Following recommendations from Fertan et al. (2019b), I observed the mice at different time points to examine age-related behavioural changes. However, since behavioural measures can be relatively variable, observing the same mice at each time point in a longitudinal study might be more beneficial than observing different groups of mice in a cross-sectional study. Nevertheless, it is rather difficult to conduct such a study, especially to 17 months, due to the increased mortality rates in older 3xTg-AD mice (Rae and Brown, 2015). In addition, repeat testing of the same animal can impact behavioural tasks, as animals will habituate and learn tasks over time, which may affect their behaviour (van Heusden et al., 2021). Indeed, it has been previously shown that a mouse model of anxiety has different whisker movements to control mice (Grant et al., 2016); therefore, an altered sensitivity to stress is likely to affect this study's results. The lack of automation of the set-up may also confound testing over different ages, while in this study all data was collected over a period of just a few days, with all the equipment kept the same throughout.

As there are clear sex differences in the behaviour (Fertan et al., 2019b; Kane et al., 2018) as well as in the vasculature of 3xTg-AD mice (progressive from 4-6

months of age, Jullienne et al., 2022), and we know that whisker movements differ between sexes in other mouse models (Garland et al., 2018; Grant et al., 2018b; Simanaviciute et al., 2020), investigating whisker movements in male 3xTg-AD mice at different ages would be beneficial. It would also be interesting to investigate whether the amyloid quantity in the barrel cortex is related to whisking impairment in 3xTg-AD mice. It has been previously demonstrated that models of cortical development disorders have whisker movement deficits (in Robo3R3−5-CKO and RIM-DKOSert models), suggesting that cortical differences can affect whisker movements (Simanaviciute et al., 2020). However, previous studies have also shown differences in whisker movements in non-neurodegenerative mouse models (MCAO model of stroke and heterozygous Reeler mice, Simanaviciute et al., 2020), which would suggest that whisking impairment is not specifically related to neurodegeneration and amyloid levels in the cortex, but likely caused by many changes in the brain.

In agreement with Simanaviciute et al. (2020), measuring whisker movements is a quick, robust and semi-automated way to capture motor, sensory and cognitive behaviours in rodents. While the qualitative scoring of whisking, spread reduction, CIA and HTA were valuable at assessing whisker behaviour, they require manual scoring and are relatively time-consuming to complete. I wanted to assess whether measuring spread automatically was a more sensitive method than manual scoring, and it has shown differences at more advanced disease stages than were found by manual scoring. Therefore, it might be worth developing ARTv2 to measure these qualitative scorings automatically – developments I describe in Section 3.5. Developing quantitative data and better analytical methods will improve the robustness of repeated testing. These findings differed from Simanaviciute et al. (2020), probably due to the difference in statistical methods. Using a linear mixed effect model is suggested for future analyses (package lme4 in R-studio, Bates et al., 2015) as was done here, which makes the most of smaller and uneven sample numbers, without assuming per-clip or per-trial independence. Small improvements in automation and analysis techniques will also help to develop whisker movements as a powerful behavioural measurement

tool, with particular benefits in capturing behavioural deficits in mouse models that reveal complex or subtle phenotypes, such as in the 3xTg-AD mouse model. Indeed, the barrel cortex has been found to contain amyloid plaques in several mouse models of AD, including Tg19959 mice at 3 months (Tampellini et al., 2010), APP transgenic mice Tg2576 at 17.5 months (Bero et al., 2011) and APP/PS1 mice at 19.5-21 months of age (Beker et al., 2012). To understand the relationship between amyloid levels and whisker movement impairments, it would be beneficial to study whisking in these mouse models.

3.5 Method development: qualitative measure replacement

Video clips which pass the criteria developed by Grant et al. (2014) are normally scored for whisking, contact-induced asymmetry, spread reduction, head turning asymmetry and look-ahead behaviours (Table 3-6), as per recommendations in Simanaviciute et al. (2020). As the scoring is done manually for each video clip, it is a time-consuming task. Therefore, these measures should be made more automated and quantitative. To adapt this scoring system, I systematically examine every qualitative measure to better understand which of these measures provide additional information to the quantitative measurements already in place, and whether those selected measures can be automated and quantified.

Table 3-6 **Qualitative scoring behaviours and score description.** CIA and HTA are only scored in clips of object exploration.

Whisking (Grant, 2012)	Contact-induced asymmetry (CIA, Grant, 2012)	Spread reduction (Grant, 2012)	Head turning asymmetry (HTA, (Grant, 2012)	'Look-ahead' (Arkley et al., 2014)
(0) No whisking (1) Retraction only (2) Protraction only (3) Retraction and protraction	(0) None (1) Increased contralateral protraction (2) Reduced protraction ipsilateral to the contact (3) Both contralateral and ipsilateral	(0) Absent (1) Reduce spread on contact	(0) Absent (1) Whisker asymmetry before a head turn	(0) Absent (1) Push-forward whiskers at higher speeds

'Look-ahead' and HTA

During the process of qualitative scoring of the 3xTg-AD mouse data set, it was difficult to find video clips that demonstrate the 'look-ahead' and HTA behaviours. It is not too surprising, considering that 'look-ahead' requires high-speed running behaviour and, so far, has only been shown in rats (Arkley et al., 2014) and European dormouse (*Muscardinus avellanarius*), Etruscan shrew (*Suncus etruscus*), wood-mouse (*Apodemus sylvaticus*) and yellow-necked mouse (*Apodemus flavicollis*) species (Grant et al., 2018a), but not in house mouse (undetected in Simanaviciute et al., 2020). The current protocol of object exploration encourages mice to approach the object and that naturally slows them down, therefore, 'look-ahead' will not be present in these clips. It is more likely to detect 'look-ahead' behaviour in the open field clips, but the probability is still low as only a little over a second of footage is collected, and mice are not trained to run in the current set up – unlike the protocol of Arkley et al. (2014), who trained rats to run around a looped experimental arena.

Similarly, HTA is not encouraged by this protocol, as the focus is placed on capturing mice walking straight to the object rather than turning. Both 'look-ahead' and HTA were scored prior to contact in Simanaviciute et al. (2020) in a wide range of mouse models, yet these were removed from their analyses due to being not common. There were no occurrences of 'look-ahead' and no differences in HTA found in the 3xTg-AD mice compared to the wildtype mice. Therefore, I suggest that 'look-ahead' and HTA are not the most important measures to focus on while using the current protocol of whisker tracking and do not warrant the effort of manual scoring or quantification of these behaviours. From now on 'look-ahead' and HTA are not included in the analyses within this thesis.

Spread reduction

This 3xTg-AD dataset is the first time where spread was fully quantified in the whisker detecting software ARTv2 (Gillespie et al., 2019). Spread reduction behaviour can then be quantified by calculating (PC-DC) spread, which shows the change in spread from pre-contact to during-contact. In the 3xTg-AD mice data set, most mice consistently reduced spread upon contact (the expected behaviour, 95.58% of clips) and, therefore, spread reduction score 0 (not reduced, n=8 clips) and 1 (reduced, n=173 clips) cannot be statistically compared. However, I am confident in quantifying spread reduction this way, because it shows that 12.5-month mice are not always reducing the spread compared to other ages – Figure 3-7, in agreement with the qualitative data in Figure 3-2. Interestingly, quantifying spread reduction reveals that there might not be such a significant difference between the 12.5-month wildtype and transgenic mice, as suggested by manual scoring in Figure 3-2C, and perhaps also a stronger difference between genotypes at 17 months. However, it is difficult to know with this data set having so few clips where spread was not reduced. It would be beneficial to test if the (PC-DC) spread corresponds to the spread reduction scores in another data set where spread reduction occurs less; perhaps in a DAT knockout mouse model of attention-deficit hyperactivity disorder, which was found to exhibit hyperactivity in an open field as well as working memory and reversal learning deficits (Kantak, 2022). Studying

whisker movements in DAT knockout mice and investigating spread reduction measure could help to distinguish between attentional and memory deficits found by other behavioural tasks.

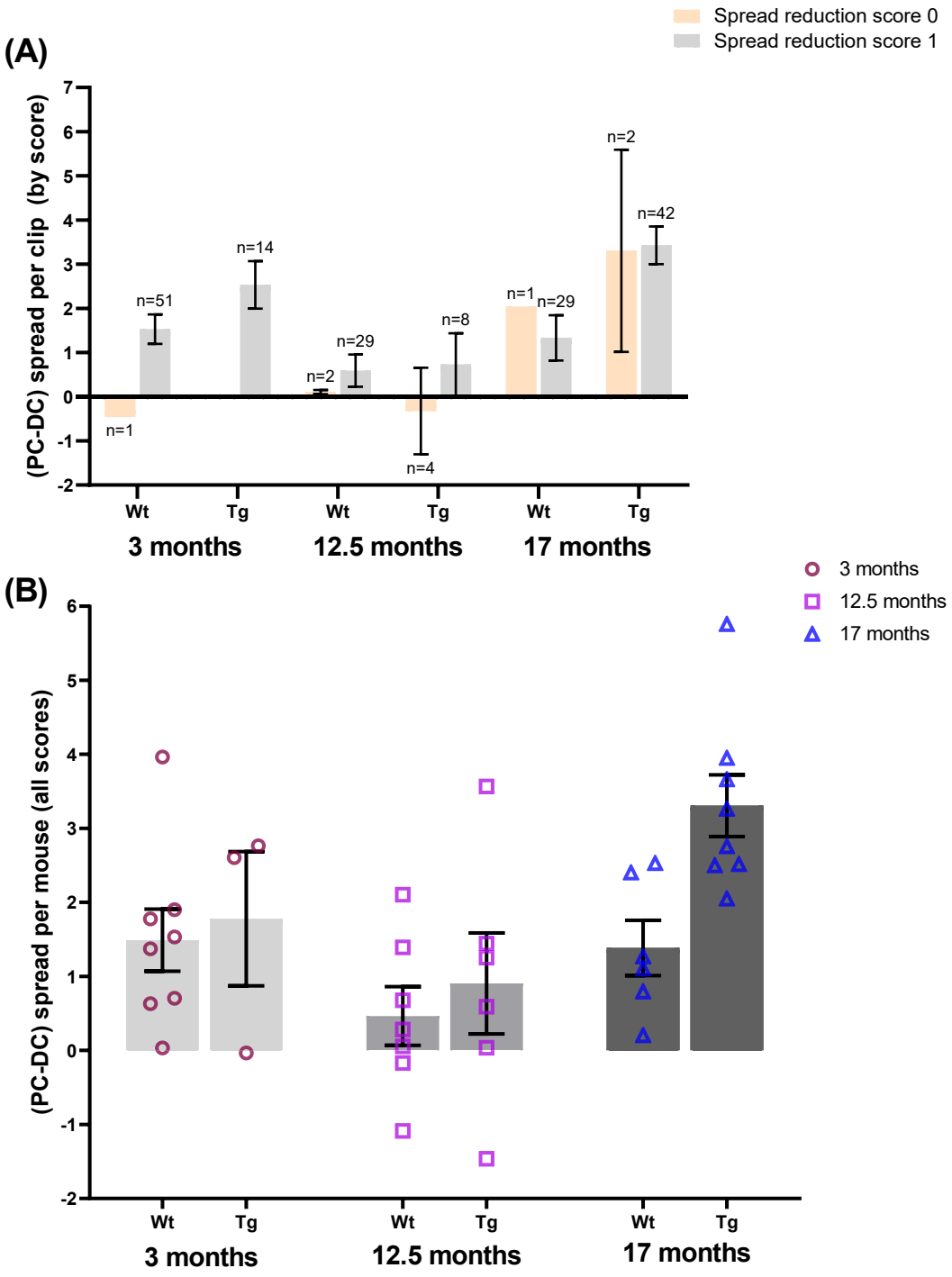


Figure 3-7 **(A) (PC-DC) spread reduction by score compared to (B) (PC-DC) spread means per mouse.** In (A), bars consist of (PC-DC) spread separated for spread reduction score 0 and 1 and n number indicates the number of clips in each score group. In (B), bars show the mean (PC-DC) spread per mouse with individual data points. Error bars show standard error of the mean in both graphs.

If spread is reduced upon contact (score 1), (PC-DC) spread values will be higher and positive, while if spread is not reduced (score 0), (PC-DC) spread is expected to be closer to 0 or even negative. Figure 3-7A shows that (PC-DC) spread is negative or very close to 0 in at least 7 out of 10 video clips where spread was scored as not reduced, and all score 1 values are positive. The same (PC-DC) spread, but per mouse, is visualised in Figure 3-7B, demonstrating a quantitative approach to studying spread reduction. This figure shows the few mice that did not reduce spread or reduced the spread only slightly were from the 12.5-month group, in both wildtype and transgenic mice. I recommend quantifying spread reduction as (PC-DC) spread and graphing the values per mouse, which allows to see any outliers and the exact number of mice in the group that are consistently not reducing the spread.

Contact-induced asymmetry

CIA can be a useful measure to score as seen in the 3xTg-AD dataset (Figure 3-2 for CIA, Figure 3-6 for quantitative asymmetry) as well as in Simanaviciute et al. (2020) that there can be differences in CIA which are not picked up in the quantified asymmetry measure. However, CIA is more complicated than other qualitative measures because not every contact can be scored for this behaviour. To determine CIA score, the contact must be unilateral (Mitchinson et al., 2007, 2011), meaning it needs to clearly come from one side rather than from straight ahead. However, I observe that contacts tend to be bilateral in most cases (53%). Therefore, a lot of the clips collected are not suitable for CIA scoring, because the set up encourages clip collection when mice to go straight to the object. Since CIA is contact-related behaviour and can only be scored in the object exploration task, there are generally not enough clips to reliably perform statistical analysis on, whether that is analysing qualitative scores or trying to quantify CIA. Moreover,

quantifying CIA requires additional input of the side of contact to determine whether the contralateral angle increased and ipsilateral angle decreased. Considering all the points raised, I suggest that CIA is not a great measurement to be used with the current tracking protocol and that any additional differences found in CIA scores that do not show up in quantitative measures can be inaccurate, depending on the set up and the experience of the scorer.

Whisking scores

The whisking scores applicable to my research are often only scored 2 and 3, as score 1 (retractions only) occurs mostly in very young mice which are not fully developed (Grant et al., 2012), while score 0 has never occurred in the datasets before (including in Simanaviciute et al., 2020). Nevertheless, I considered quantifying whisking scores as they have previously revealed differences between transgenic or surgery-induced disease models; specifically, R6/2 CAG250 mouse model of Huntington's disease was different from all other disease models tested in Simanaviciute et al. (2020). It is expected that score 2 would show higher minimum angles and score 1 would show lower maximum angles than the "mean" angle. However, the threshold mean angle for these comparisons would have to be determined for each data set. I have compared the minimum and maximum angles in scores 2 and 3 to identify any obvious measures that might show differences between these scores (Figure 3-8A-B). The minimum whisker PC angle revealed significant difference between whisking score 2 and 3 in the 17-month transgenic mice, suggesting that the minimum whisker angle could be used to describe whisking capacity in quantitative terms. Alternatively, PC whisker amplitude and mean angular position together can be a good proxy for evaluating whisking capacity and does show very similar differences as the qualitative whisking score.

Furthermore, Simanaviciute et al. (2020) report that transgenic R6/2 CAG250 mice, which had reduced whisking scores compared to other transgenic mice, also had increased PC whisker mean angular position and smaller amplitudes (Garland et al., 2018), supporting the suggestion that amplitude and mean angular position

can be used together to replace the current whisking scoring. In the current data set of 3xTg-AD mouse model, the 17-month transgenic mice show reduced whisking scores (Figure 3-2A, Figure 3-8B) and reduced PC amplitude as well as PC mean angular position (Figure 3-8C-D). Unfortunately, there are not enough video clips to show this in the 12.5-month group as the 12.5-month wildtype mice whisked fully in 30 out of 31 clips and it was not possible to run any statistical analysis with such low sample size. However, Figure 3-8A-B shows that the 12.5-month transgenic group had more 2 scores than 3 scores and that would naturally be significantly different from the wildtype group, as evident when looking at the quantified PC amplitude and PC mean angular position (Figure 3-8C-D). Hence, whisking scores can be assessed accurately by looking at the quantitative measures of amplitude and mean angular position, and it is not recommended for manual scoring in future studies.

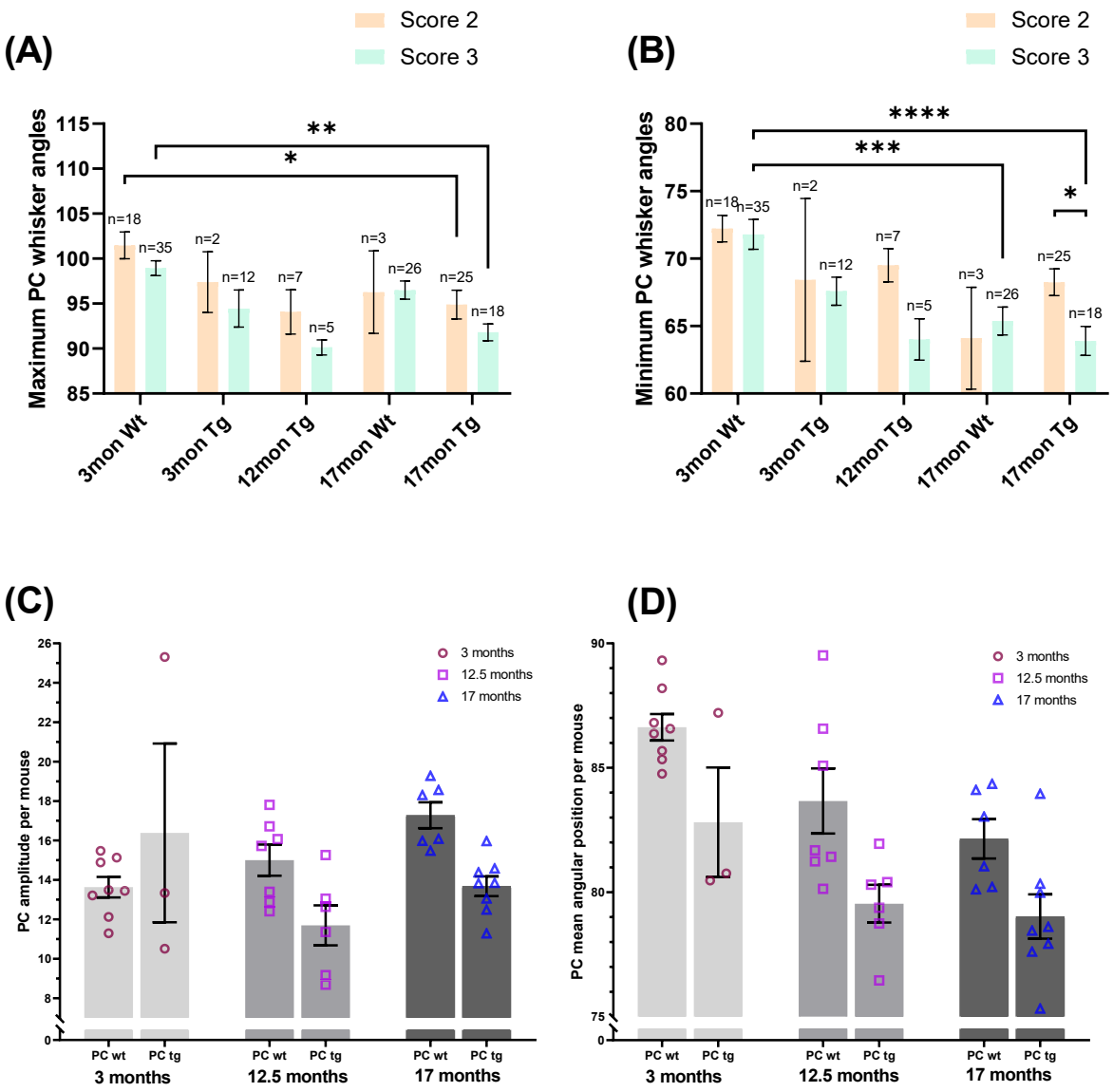


Figure 3-8 **Maximum (A) and minimum (B) whisker PC angles in comparison with PC amplitude (C) and PC mean angular position (D).** The minimum PC whisker angles show significant difference between scores 2 and 3 in the 17-month transgenic mice, suggesting that more mice in that group had reduced whisking capacity. The same can be seen by studying PC amplitude and PC mean angular position.

Relationship between the quantitative whisker measurements

To understand if all quantitative whisker parameters provided by the ARTv2 tracker are necessary to include in analysis, I performed a correlation analysis on whisker parameter data (without locomotion speed) from Chapter 3, using the *cor* function from the R *stats* package. In the resulting correlation matrix, where each whisker variable is compared to another (e.g. PC spread from all mice against PC asymmetry from all mice), several correlations appeared. For example, PC spread was correlated with PC amplitude but not to PC asymmetry. Meanwhile, DC spread was correlated to both DC asymmetry and DC amplitude (Figure 3-9). From the correlation matrix, the following relationships stood out due to their low p-values and high correlation coefficients:

1. PC spread vs PC amplitude, $p < 0.001$, negative correlation, coefficient = -0.616;
2. DC asymmetry vs DC spread, $p < 0.001$, negative correlation, coefficient = -0.574;
3. DC asymmetry vs DC amplitude, $p < 0.001$, positive correlation, coefficient = 0.627.

Thus, I looked at these same correlations in more detail, this time plotting them per animal. The linear model analysis (*lm* function, R *stats* package) confirmed the existence of a relationship between these variables as indicated by the p-values. However, the adjusted R^2 values were close to 0, suggesting a linear model is not the best fit for these relationships (Figure 3-10, Figure 3-11 and Figure 3-12).

Hence, the answer to whether two whisker variables are linearly related to each other in the same animal is not straightforward. Naturally, as these parameters all stem from whisker angles and distances, they are overall correlated. However, they are not always correlated in the same animal, as showed by the linear models; for example, a high DC asymmetry value does not always correspond to a low DC spread value (Figure 3-9).

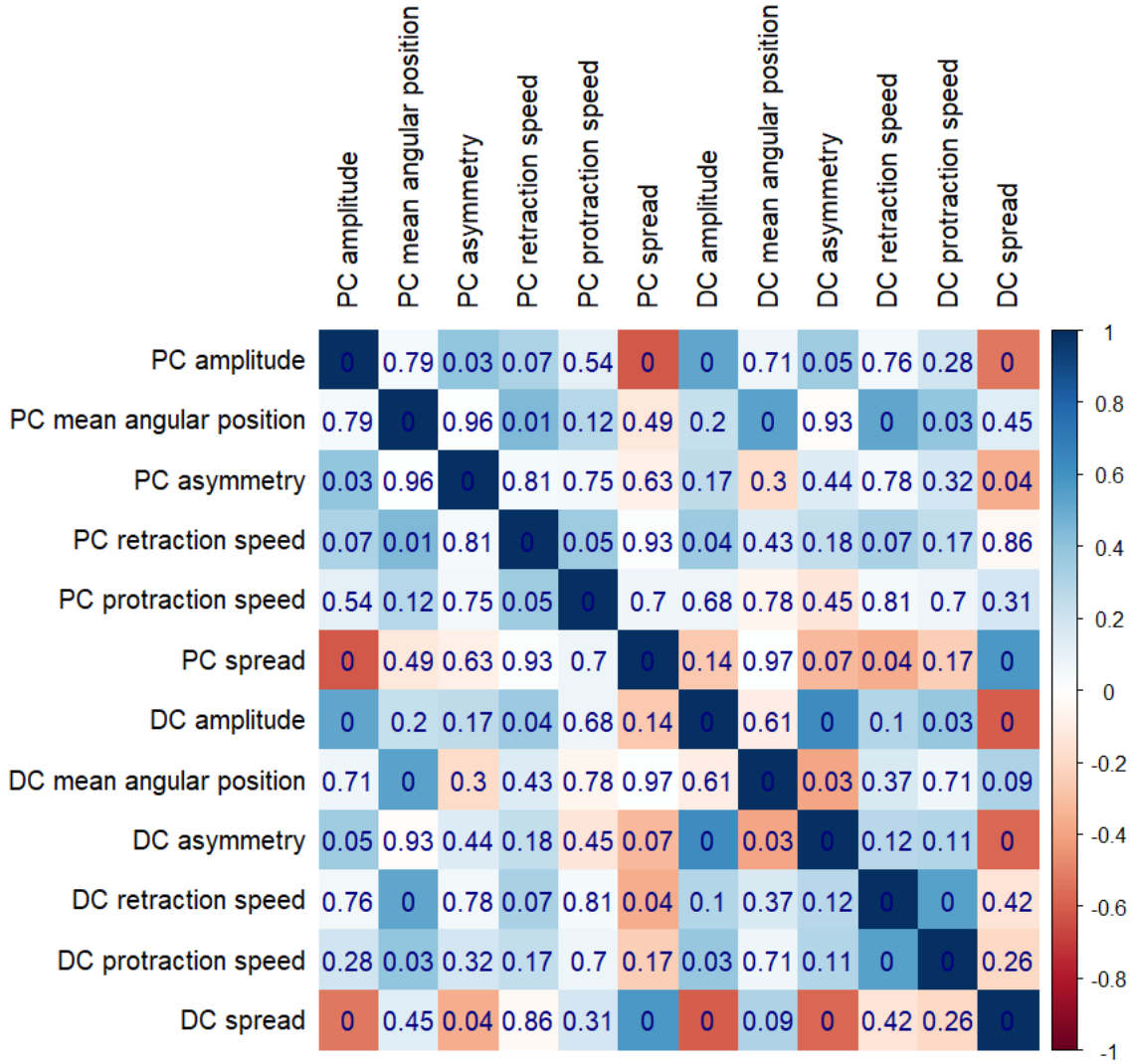


Figure 3-9 **Correlation matrix for all whisker parameters measured in Chapter 3.** The matrix shows all p-values for the correlation between the corresponding parameter pairs. A significance value of $p < 0.05$ was used. If $p < 0.01$, it is shown as zero. The colour scale uses a correlation coefficient between –1 and 1. Positive linear correlation is represented with shades of blue, with a coefficient of 1 and dark navy shade illustrating a perfect positive linear relationship; negative linear correlation is represented with shades of red, with a coefficient of –1 and dark red shade illustrating a perfect negative linear relationship. The zero value on the colour scale, corresponding to the colour white, illustrates no correlation.

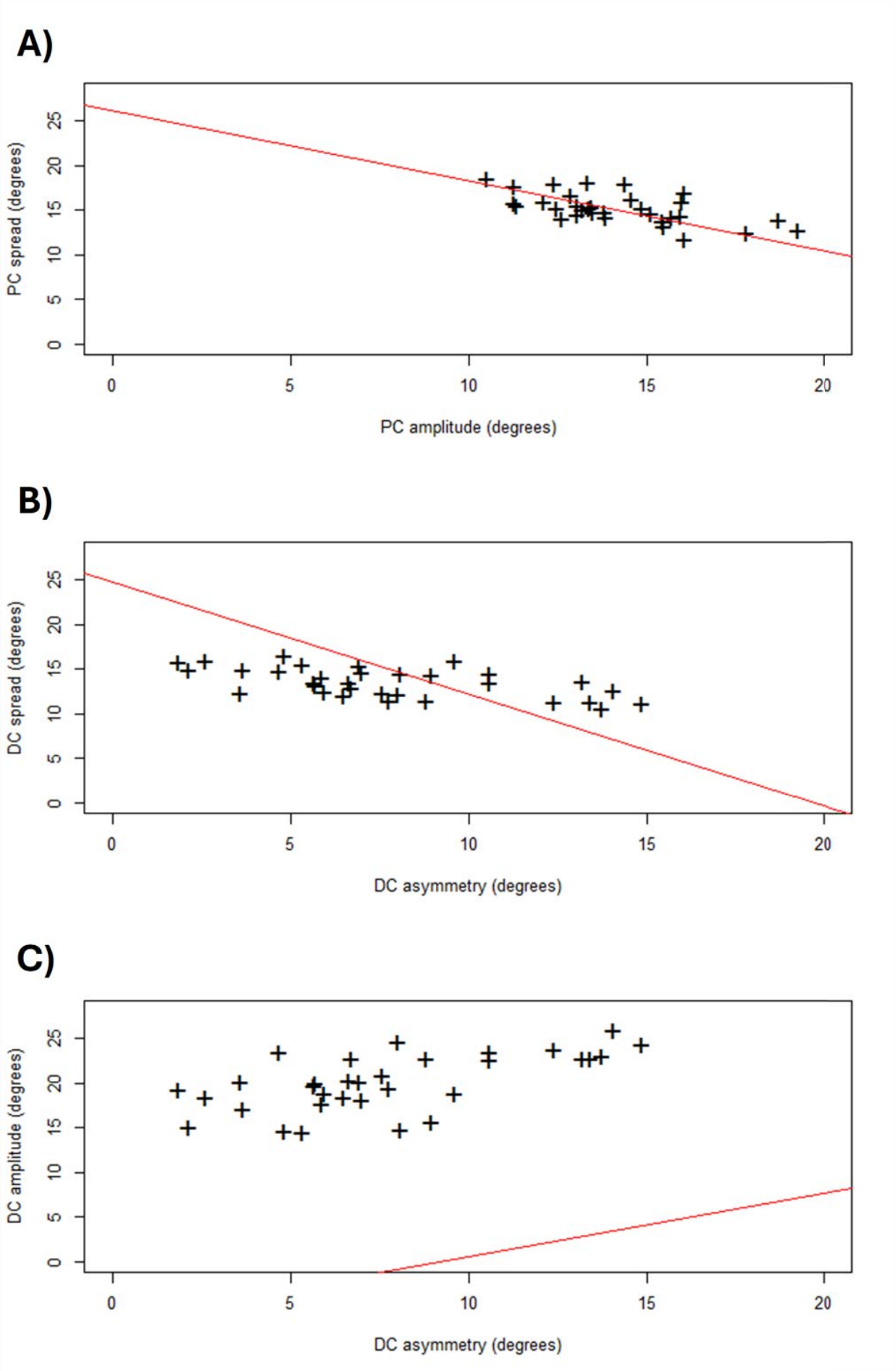

A)
PC spread (degrees)
0
5
10
15
20
25
0
5
10
15
20
PC amplitude (degrees)
B)
DC spread (degrees)
0
5
10
15
20
25
0
5
10
15
20
DC asymmetry (degrees)
C)
DC amplitude (degrees)
0
5
10
15
20
25
0
5
10
15
20
DC asymmetry (degrees)

Figure 3-10 **The correlation between whisker parameters per animal.** A - PC amplitude vs PC spread, *adjusted R^2 = 0.3588*; B – DC asymmetry vs DC spread, *adjusted R^2 = 0.307*; C - DC symmetry vs DC amplitude, *adjusted R^2 = 0.373*. Values are plotted for each animal (represented with +). The red line shows a linear model fitted to the data – all *p values < 0.001*. This plot includes all animals studied in Chapter 3: female control and 3xTg-AD mice of 3-, 12.5- and 17-month age groups; *n = 38.*

From these results, I recommend that all whisker parameters are continued to be measured, and I do so in this thesis, until more detailed studies show otherwise, and provide which measurements are strongly related and if any may be unnecessary. Specifically, relationships should be tested in both pre-contact and contact-related whisker parameters, as this preliminary analysis shows correlations differ between the two. Moreover, I acknowledge that my chosen approach is not the only option and other methods, such as clustering analysis, could be used, as mentioned in Chapter 6 Discussion – Limitations and future work.

3.6 Conclusion

I have validated the current protocol in a challenging mouse model of Alzheimer's disease. This work has, for the first time, established statistical analysis methods that are more suitable for the measures captured by the protocol. I have also introduced an additional quantitative measure of whisker spread and replaced qualitative scoring of spread reduction and whisking with a quantitative (PC-DC) spread measure, PC whisking amplitude and PC mean angular position. This results in a more streamlined process of whisker tracking and analysis and makes the process more quantitative and objective. The work in this chapter contributes to the first two aims of the PhD – standardisation and automation.

CHAPTER 4 MATERNAL IMMUNE ACTIVATION AFFECTS FEMALE OFFSPRING WHISKER MOVEMENTS DURING OBJECT EXPLORATION IN A POLY I:C RAT

This chapter has been published as a peer-reviewed journal article and included in the appendix of the thesis:

Simanaviciute, U., Potter, H.G., Hager, R., Glazier, J., Hodson-Tole, E., Gigg, J., Grant, R., 2024. Maternal immune activation affects female offspring whisker movements during object exploration in a rat model of neurodevelopmental disorders. Brain, Behavior, & Immunity - Health 39, 100807.

Chapter summary

Neurodevelopmental disorders (NDDs) can be highly disabling, causing reduced life expectancy and disturbing physical and cognitive symptoms. The maternal immune activation (MIA) rat model, induced by an environmental risk factor polyinosinic:polycytidylic acid (Poly I:C), is a model of neurodevelopmental disorders. The behavioural symptoms of this model are diverse; they are often variable and reduce in severity or disappear when offspring reach adulthood. It is important to select a robust behavioural test to identify such subtle phenotypes, track progression into the long-term and develop treatments. Measuring whisker movements presents an opportunity to study innate exploratory rodent behaviour, resulting in highly quantitative, robust measurements of sensory, motor and cognitive behaviours but it is missing cross-species validation. Tracking whisker movements in adult MIA offspring may offer further detail and reveal robust and long-lasting behavioural deficits. In this study, whisker movements were investigated in adult male and female offspring of MIA-exposed rat dams and compared to age-matched offspring of control (vehicle) dams. Rat offspring were filmed using high-speed videography in a sequential object exploration task with smooth and textured objects. Poly I:C treatment effects were found in female offspring who did not increase whisker mean angular position during object exploration, indicating an attentional deficit. Sex differences and object type were

also found to significantly affect whisker movements; for example, female offspring had more symmetric whiskers on the smooth object, compared to the textured object, while male offspring did not increase their whisker mean angular positions during contact with the smooth object, as they did on the textured object. Whisker tracking in rats is demonstrated here, for the first time, during sequential object exploration as a useful, non-invasive tool, with no animal training required. I show here that it is powerful enough to detect both treatment and sex effects into adulthood, in a model of NDDs caused by MIA.

4.1 Introduction

Neurodevelopmental disorders (NDDs), including schizophrenia, attention deficit hyperactivity disorder and autism spectrum disorder, affect the development of the nervous system and normal brain function. This can have wide ranging consequences, impacting emotions, learning, self-control and memory. Schizophrenia is one of the more severe NDDs, where patients not only die on average 20 years earlier than the general population (Laursen et al., 2014) but are also affected by chronic psychosis. NDDs also affect males and females differently. For example, the age of schizophrenia onset is earlier in men, and they are more likely to experience negative symptoms and deficits in social functioning (Li et al., 2016; da Silva et al., 2023). In women, oestrogen seems to play a role; although women are generally less likely to develop schizophrenia, a second peak of onset coincides with menopause, and is also likely to be more severe (Li et al., 2916; da Silva et al., 2023).

There is currently no cure for NDDs, and a lot of effort is put into trying to understand this disease group to develop better treatments. The cause of NDDs likely involves an interaction between genetic and environmental risk factors (such as viral and bacterial infections, taking antiepileptic valproic acid and consuming alcohol, Santos-Terra et al., 2021), which affect early neurological development and result in pathological changes (Owen and O'Donovan, 2017). Epidemiological studies have shown that, in humans, prenatal exposure to inflammation *in utero* is associated with the development of NDDs in offspring, including schizophrenia

and autism, (Atladóttir et al., 2010; Brown, 2012; Jiang et al., 2016; Han et al., 2021).

Animal models

Rodent models, including rats and mice, are particularly useful in identifying symptoms and developing treatments for many neurological conditions because of their genetic similarity to humans. Historically, mice have been the preferred rodent model for most conditions, since they breed quicker and are smaller, therefore can be stored more efficiently and thus reduce the costs of research projects. However, rats tend to be favoured over mice when no genetic manipulation is involved, especially in cognitive studies as they tend to take less time to learn a task (Elenbroek and Youn, 2016). Their social behaviour is also simpler, they are less aggressive, less hierarchical and less territorial than mice, which might make them better suited for modelling diseases that affect social behaviour (Elenbroek and Youn, 2016). Particularly for neuropsychiatric disorders, the 5-HT6 subtype of serotonin receptors in areas related to the affected behaviour is more similar in rats to that of humans, compared to in the mouse brain (Elenbroek and Youn, 2016). Additionally, in rats but not in mice, 5-HT6 receptors bind some antagonists with high affinity. This is more similar to that in humans and makes rats a more translatable model animal for treatment development (Elenbroek and Youn, 2016).

In pre-clinical studies, maternal immune activation (MIA) rodent models of NDDs have been reported to be particularly strong in constructive and face validity, suggesting they reflect the natural pathogenesis of the NDDs and their symptoms (Woods et al., 2021). Recently, research using a rat MIA model has shown that placental amino acid transport function is dysregulated and may contribute to the abnormalities reported in foetal brain development, which associate with increased risk of NDDs in the offspring (Kowash et al., 2022). A widely used method to induce MIA and mimic viral infection during pregnancy is the gestational exposure to the viral mimetic and Toll-like receptor 3 agonist polyinosinic:polycytidylic acid (Poly I:C, Bucknor et al., 2022).

In Poly I:C models, offspring have demonstrated behavioural deficits in sensorimotor gating, selective attention, social behaviour, exploratory behaviour, working memory, cognitive flexibility and have increased sensitivity to psychotomimetic drugs (Meyer, 2014; Potter et al. 2023). Due to the successful replication of these cognitive and behaviour outcomes in such models, there has been considerable interest in elucidating the biological and molecular mechanisms underpinning NDDs, with the Poly I:C model already being used to explore possible therapeutic treatments (Piontkewitz et al., 2009; 2011), as recently reviewed in (Bergdolt and Dunaevsky, 2019), making it a particularly relevant model of clinical interest at this time. However, while the Poly I:C model may be preferable for developmental studies, it is a challenging behavioural model, as findings can vary depending on the various factors including the rodent genetic background, source of Poly I:C, dose and gestational timing of treatment (Mueller 2018; Kowash et al. 2019; Murray et al. 2019). Even the choice of caging system can affect maternal behaviour and the behaviour of adult offspring, including deficits in working memory, social interaction and sensorimotor gating (Mueller et al., 2018). The symptoms exhibited by offspring of MIA-affected dams in this model are diverse, but include sensory, motor and cognitive components, suggesting it could act as a more general model of NDDs. Consequently, it is imperative to select a robust and highly quantitative behavioural test to identify complex or subtle phenotypes (Simanaviciute et al., 2022), to reliably detect symptoms, track their progression and develop treatments.

Measuring whisker movements

I hypothesize that measuring whisker movements offers a robust test as a valuable alternative for studying multi-modal deficits of MIA models that are particularly difficult to detect in adulthood using standard behavioural tests. Indeed, measuring rodent whisker movements has previously revealed motor, sensory and cognitive deficits in mouse models of neurodegenerative disease (Grant et al., 2014; Garland et al., 2018; Simanaviciute et al., 2020). Whisker movements are especially useful at identifying behavioural deficits in challenging

rodent models with complex behavioural phenotypes, such as the 3xTg Alzheimer's Disease mouse model (Simanaviciute et al., 2022 and Chapter 3 of this thesis), and has been found to identify behavioural phenotypes earlier than any other behavioural test, e.g. in a R62 Huntington's Disease mouse (Garland et al. 2018).

Measuring whisker movements during object exploration provides a tool which can identify sensory and attentional deficits, previously documented in mouse models of Alzheimer's disease (5xFAD mice in Grant et al. 2018; 3xTg-AD mice in Simanaviciute et al., 2022 and Chapter 3). Whisker positioning is thought to effectively reflect an animal's attentional state (Mitchinson et al., 2007; Arkley et al. 2014) and impaired attention has been suggested as a subtype of schizophrenia for genetic studies (Cornblatt et al., 2001). Most standard behavioural tasks that measure attention involve extensive training of animals and use food as motivation, whereas whisker tracking allows the measurement of attention and sensorimotor functions as part of an animal's innate exploratory behaviour, without any previous training. Therefore, measuring whisker movements offers a quantitative, relatively non-invasive and quick method to assess behavioural deficits. However, rat models have not been investigated so far, apart from in the sub-chronic PCP rat model of schizophrenia (Landreth et al., 2021) where their whisker movements were not found to be impacted.

Whisker movements have also revealed sex differences in previous studies in mouse models of Alzheimer's disease (Grant et al., 2018) and Huntington's disease (Simanaviciute et al., 2020), which makes it especially aligned for the study of NDDs. Indeed, Lins et al. (2019) recommend investigating sex by treatment analyses in MIA research, not only because it is important to include both males and females in all preclinical research, but also because NDDs exhibit sex-dependent phenotypes in rat models (Snigda et al., 2011, Leger and Neill, 2016; Nikolić et al., 2017; Casquero-Veiga et al., 2023, Potter et al., 2023) and in humans (Li et al., 2016; May et al., 2019; Bucci et al., 2023; da Silva et al., 2023; Bölte et al., 2023). Therefore, whisker tracking could be a valuable tool for identifying sex differences in NDD rodent models.

This study aims to test and validate the established mouse whisker measurement protocol (Simanaviciute et al., 2020; 2022) in the rat Poly I:C model, to investigate the effects of *in utero* exposure to MIA on whisker movements in adult offspring. The inclusion of a larger sample size of males and females is important for the method development due to generous size differences between the sexes. The use of a sequential object study, as was performed in mice in Grant et al. (2018), allows to explore how the order or texture of objects can influence whisker movements.

Whisker movements were investigated before and during object exploration, and differences were examined by treatment, sex and responses to object textures. I predict that measures of whisker movements will be sensitive enough to detect treatment and sex effects in this adult behavioural model of NDD.

4.2 Materials and methods

Animals

The rat MIA model used here has recently been described in Potter et al. (2023). Specifically, pregnant Wistar female rats (Charles River Laboratories, UK) were injected with 10 mg/kg bodyweight low molecular weight poly I:C (InvivoGen, France, catalogue code tlrlpicw) or equivalent volume of endotoxin-free physiological 0.9 % saline on gestational day 15, where the day of mating (indicated by visualisation of the copulation plug) was designated as gestational day 1. This source of poly I:C (Kowash et al., 2019) and genetic background (Murray et al., 2019) were found to produce the most robust maternal inflammatory phenotypes (Lorusso et al., 2022; Kowash et al., 2022, Potter, 2023). Dams were pseudo-randomised to treatment group using a random number generator (Excel v2004, Microsoft, USA). MIA effect was validated by taking a tail vein blood sample at 3 h post-treatment and plasma samples analysed as per Kowash et al., (2022). The offspring of these rats were assessed for whisker movements once they reached early adulthood at around postnatal day (PD) 50 (47-53), with a bodyweight of 162 ± 3 g for males and 142 ± 2 g for females (measured at PD35, n = 25 for both males and females, mean ± SEM, Table 4-1). At this age, rat whisker movements are considered to be adult-like (Grant et al. 2012). From a total of 24 MIA offspring rats, 11 were female and 13 male, and from 26 control rats, 14 were female and 12 male. Both control and poly I:C rat offspring came from 11 dams each. Sample sizes were calculated using the statistical package G*Power v3.1.9.2 (Germany, Faul et al. 2007), based on similar previous studies (Potter et al. 2023). Half of the rats were cross fostered as part of a satellite study measuring effects of cross fostering (randomised using a random number generator); however, this had no significant effect on whisker measures (Supplementary material, Table 4-5 and 3-7) nor on other adult behaviours (Potter, 2021). Therefore, data were combined, and cross-fostering status was not included in analyses. Adult offspring were housed in cages of up to 3 females or 5 males, in environmentally enriched, individually ventilated cages (GR1800 Double-

Decker Cage, Tecniplast, UK, temperature 21°C± 2°C, humidity 55 ± 5%) with *ad libitum* access to standard rodent chow (Special Diet Services, UK) and water.

Experimental procedures

All experimental procedures were carried out at the University of Manchester Biological Services Facility, on the 12-hour light:dark cycle, lights on at 7:00am). Experiments were performed under Home Office UK project licence (number P473EC3B1) in accordance with the Animals (Scientific Procedures) Act UK 1986. This study was approved by the University of Manchester Animal Welfare and Ethical Review Body as well as the local ethics committee at Manchester Metropolitan University (reference number 2022-46121-37567). Before being involved in this study, all animals had been exposed to other tasks at PD35 (~15 days before this study, as described in Potter et al., 2023), including novel object recognition (NOR), elevated plus maze and social interaction tasks; although these tasks all used different arenas and objects to those adopted in this study.

Rats explored two different objects sequentially for 5 minutes each. Firstly, the smooth plastic toy brick (14.3 cm × 6 cm × 3 cm; Figure 4-1B) was placed inside the arena for exploration, then later exchanged for the textured toy brick (identical to smooth object but covered in textured painter's tape). This order was kept throughout the experiments and from here onwards, the first object is described as 'smooth' and the second as 'textured'. For all other methods which remain unchanged across the experimental Chapters, please refer to Chapter 2 General methods.

Video clip analysis of whisker movements

When using this set up in mice, locomotion speed can be estimated by tracking the centroid of the body. However, due to the larger size of rats compared to mice, there were very few frames where the rat's full body was in view; therefore, the centroid position was not accurate, and locomotion speed was not analysed in this study. Rats have up to double the whisker length compared to mice; whisker morphologies are heavily correlated to the head size and the distances between facial features, which are approximately 2.03 times larger in rats compared to mice (Bresee et al., 2023). While I did not measure whisker or body lengths in my studies, rats in Chapter 4 were visually more than double the size and length than mice in any earlier study I conducted. Moreover, there was a 6-10-time difference in body weight between control female mice in Chapter 3 (~22g at 10 weeks old, The Jackson Laboratory, n.d.) and control female rats in Chapter 4 (~144g 15 days before filming whisker study, see Table 4-1; ~264g at the end of the study as in Potter et al. 2023). There are also camera and tripod length limitations that need to be mentioned as I did not try to use a different camera or tripod to record the rats - this may be a solution for the future. The 6-10-time body mass difference compared to up to 2 times whisker length difference can explain why it was difficult to keep the focus on rat whiskers and consistently include the whole of rat's body in the field of camera's view. Hence the centroid speed was excluded from the measurements.

1-4 video clips per rat were included in data analysis (Table 4-1), giving a total of 114 clips, which included both PC and DC sections. PC sections ranged from 100 to 291 frames per clip, whereas DC sections ranged from 100 to 459 frames.

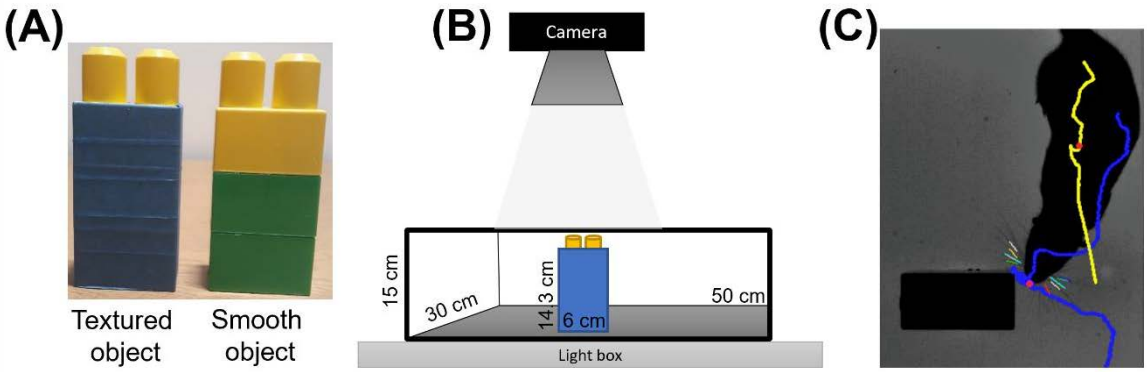


Figure 4-1 **Data collection and video analysis.** A) Filming set-up illustrating the object size and location in relation to the Perspex box, and the distance between the arena and high-speed video camera. B) Smooth and textured plastic bricks that were used as objects in the experiments; C) A video still of an example clip where ARTv2 LocoWhisk software was used to automatically locate the rat centroid and nose point which are marked by the two red points. The paths taken by these two points over the clip duration are shown in yellow (centroid) and blue (nose point) lines. The software also detected whiskers (shown with coloured lines). The field of view shown in A in light grey corresponds to this video still.

Table 4-1 **Number of videos per rat included in analyses.** Sex abbreviated: F – female; M - male. Treatment: Vehicle - injection of 0.9 % saline; Poly I:C - injection of polyinosinic:polycytidylic acid to rat dams used in the study (induces the MIA model). Note that the smooth object was also always the first object, and the textured object was always the second object presented to rats.

Rat ID	Sex	Treatment	Body weight at PD35 (g)	Clips with smooth object	Clips with textured object
551	F	Poly I:C	136.2	0	1
552	F	Poly I:C	148.3	1	2
553	F	Poly I:C	138.5	2	1
554	F	Vehicle	134.5	2	2
601	M	Poly I:C	133.3	0	2
602	M	Vehicle	166.3	0	2
603	M	Vehicle	167.7	4	1
604	M	Vehicle	164.2	0	1
431	F	Poly I:C	148.6	1	1
555	F	Vehicle	172.3	1	2
611	M	Poly I:C	167.4	3	0
561	F	Vehicle	133.1	0	3
562	F	Poly I:C	125.8	0	2
563	F	Vehicle	123.5	1	0
612	M	Vehicle	192.0	0	1
613	M	Poly I:C	150.6	1	1

614	M	Vehicle	148.2	1	0
621	M	Poly I:C	152.4	0	1
632	M	Poly I:C	168.9	2	2
564	F	Vehicle	161.8	0	2
565	F	Vehicle	161.2	1	0
573	F	Vehicle	139.3	0	2
574	F	Poly I:C	148.7	1	1
622	M	Vehicle	177.0	1	2
623	M	Poly I:C	146.0	0	2
633	M	Vehicle	186.7	1	0
634	M	Poly I:C	140.9	0	4
571	F	Vehicle	147.4	1	3
581	F	Vehicle	142.5	2	0
624	M	Vehicle	157.8	1	1
631	M	Poly I:C	161.0	2	0
642	M	Vehicle	158.1	2	0
575	F	Vehicle	142.1	1	2
582	F	Vehicle	134.2	0	1
583	F	Poly I:C	139.8	1	0
641	M	Poly I:C	182.5	0	1
643	M	Vehicle	153.1	1	2
644	M	Poly I:C	149.5	3	1
584	F	Poly I:C	138.6	0	1
585	F	Poly I:C	131.5	0	4
591	F	Vehicle	133.4	0	1
592	F	Vehicle	145.4	1	0
593	F	Poly I:C	138.6	2	2
594	F	Vehicle	139.4	0	3
595	F	Poly I:C	153.1	0	2
651	M	Vehicle	173.2	0	1
652	M	Poly I:C	164.3	0	1
653	M	Poly I:C	157.7	2	1
654	M	Vehicle	161.7	2	1
655	M	Poly I:C	169.9	1	3
	Total rats: 50 MIA offspring rats n = 24 (11 female, 13 male) control rats n = 26 (14 female, 12 male)		Mean weight (g): Control female 143.6 ± 3.6; Control male 167.2 ± 3.8; Poly I:C female 140.7 ± 2.5; Poly I:C male 157.3 ± 3.8	Total clips: 114 45 clips (29 rats) with smooth object	69 clips (40 rats) with textured object

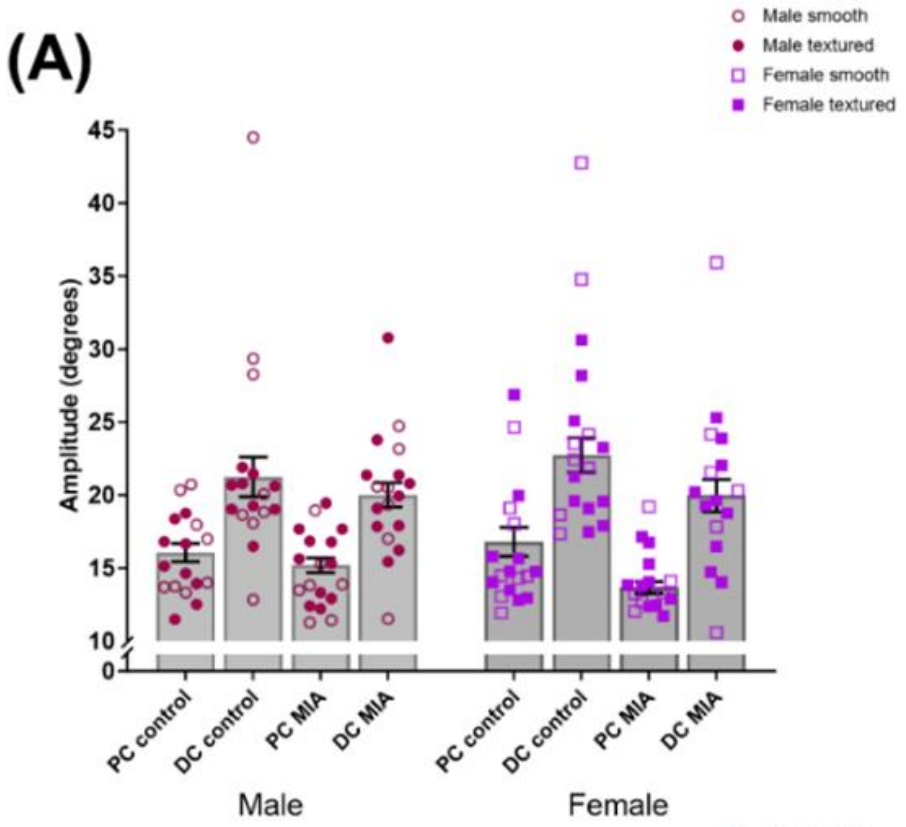
Statistical analyses

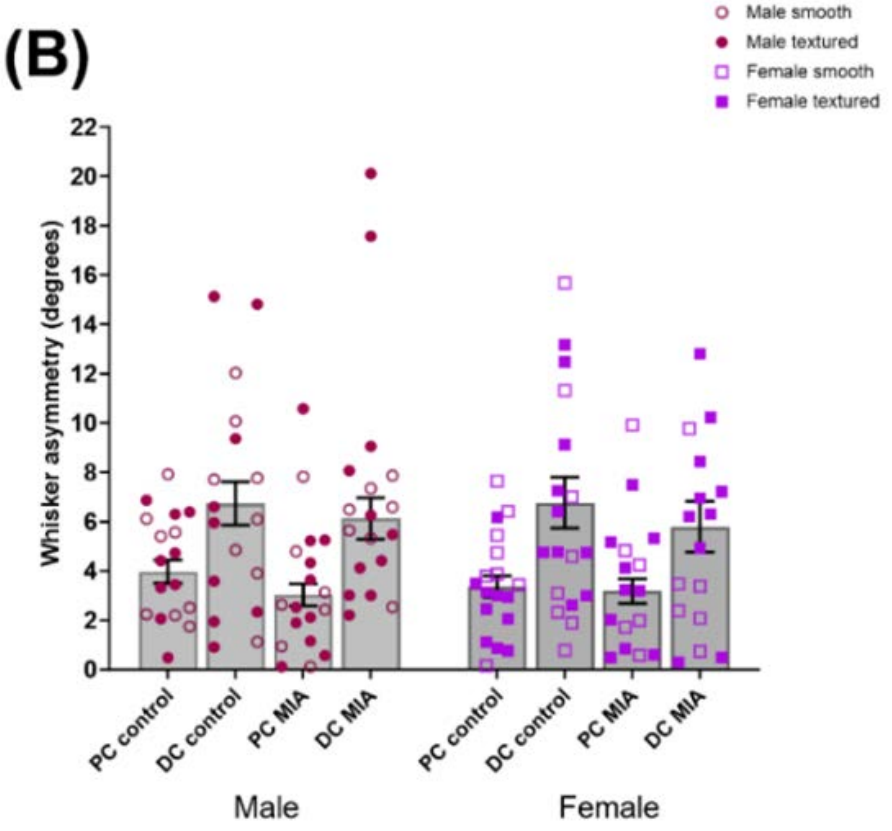
Linear Mixed-Effects Models were constructed to analyse the effect of Poly I:C treatment, sex and object texture or order on all PC and (PC-DC) whisker variables. The model computed F tests on the fixed effects of treatment, sex and object texture or order and provided *p*-values using a type III ANOVA, as well as interaction effects. The degrees of freedom were automatically determined to be anywhere between the number of rats (n = 50) and the number of video clips (n = 114) for each measurement analysed. The effect of Poly I:C treatment and sex, as well as their interaction, was investigated first. Then, males and females were assessed separately for the effect of treatment and object texture.

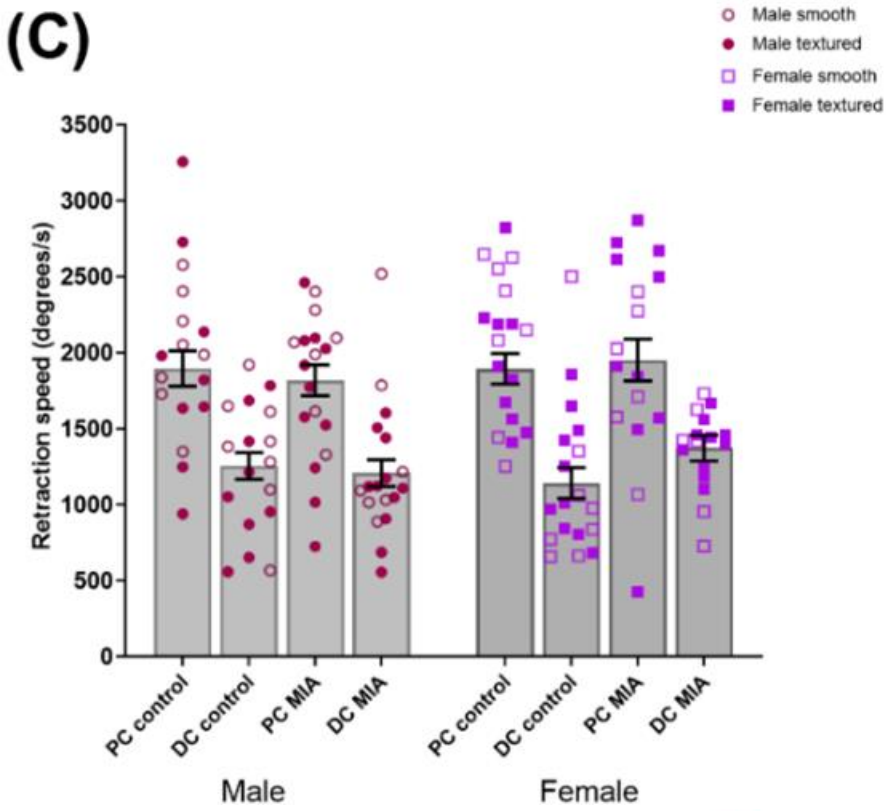
4.3 Results

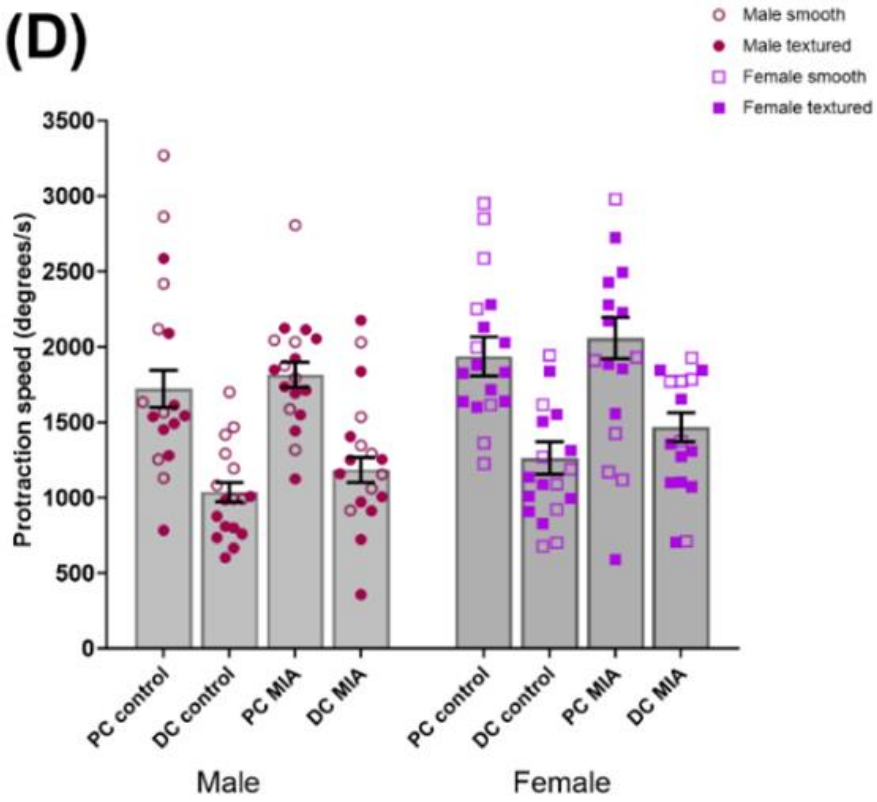
Robust behaviours displayed by both MIA and control offspring rats

In analyses with males and females combined, both MIA-treated and control rat offspring exhibited the predicted, robust contact-related behaviours that have been previously observed in mice (as described in Table 2-1). During contact with an object, whisker retraction speed, protraction speed and spread were consistently reduced (Figure 4-2C, D, and E, respectively, indicated by the difference between PC and DC values), while amplitude and asymmetry increased (Figure 4-2A, B, respectively, indicated by the difference between PC and DC values). It is important to consider the presence or absence of these behaviours when interpreting any significant differences caused by the treatment, sex, or object texture in PC-DC measures.


(A)
Male smooth
Male textured
Female smooth
Female textured
Amplitude (degrees)
45
40
35
30
25
20
15
10
0
PC control
DC control
PC MIA
DC MIA
PC control
DC control
PC MIA
DC MIA
Male
Female


(B)
Male smooth
Male textured
Female smooth
Female textured
Whisker asymmetry (degrees)
22
20
18
16
14
12
10
8
6
4
2
0
PC control
DC control
PC MIA
DC MIA
PC control
DC control
PC MIA
DC MIA
Male
Female


(C)
Male smooth
Male textured
Female smooth
Female textured
Retraction speed (degrees/s)
3500
3000
2500
2000
1500
1000
500
0
PC control
DC control
PC MIA
DC MIA
PC control
DC control
PC MIA
DC MIA
Male
Female


(D)
Male smooth
Male textured
Female smooth
Female textured
Protraction speed (degrees/s)
3500
3000
2500
2000
1500
1000
500
0
PC control
DC control
PC MIA
DC MIA
PC control
DC control
PC MIA
DC MIA
Male
Female

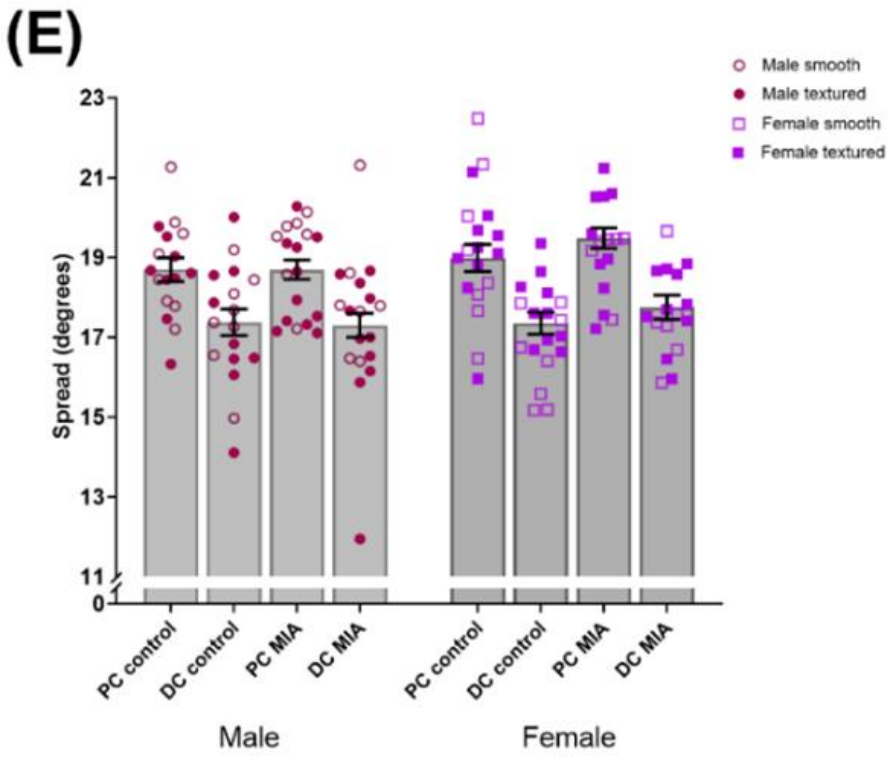


Figure 4-2 **Poly I:C treatment, sex and object texture (or order) did not have significant effects on whisker amplitude, asymmetry, protraction speed, retraction speed or spread, when analysed with male and female rats combined.** The bars indicate the mean values from all the clips (degrees of freedom calculated from a linear mixed-effect model), with bars representing SEM. Data points show mean values for individual rats, indicated by open circles for male rats investigating smooth (first) object, filled circles for male rats investigating textured (second) object, open squares for female rats investigating smooth (first) object, and filled squares for female rats investigating textured (second) object. PC = pre-contact, DC = during contact.

Next, Poly I:C treatment and sex effects were tested. There was no effect of treatment, but a sex effect in PC mean angular position was detected (Figure 4-3A, Table 4-2). Female rats had higher PC mean angular positions than male rats (treatment: F (1, 41.224) = 2.446, p = 0.125; sex: F (1, 41.224) = 5.240, p = 0.027; interaction: F (1, 41.224) = 1.034, p = 0.315), although post-hoc analyses were not significant. There were no further significant effects of treatment or sex found in any of the other whisker variables (PC amplitude, PC asymmetry, PC spread, PC retraction speed or PC protraction speed, nor (PC-DC) mean angular position, (PC-DC) asymmetry, (PC-DC) spread, (PC-DC) retraction speed or (PC-DC)

protraction speed, Figure 4-2 for all whisker parameters, Table 4-2 for PC measures, Table 4-3 for (PC-DC) measures).

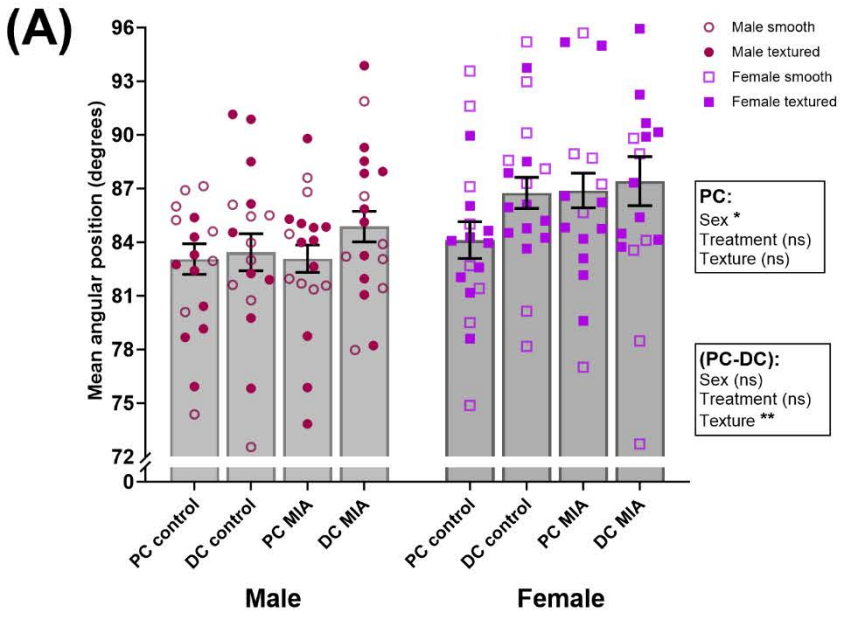
MIA treatment effects in female offspring rats

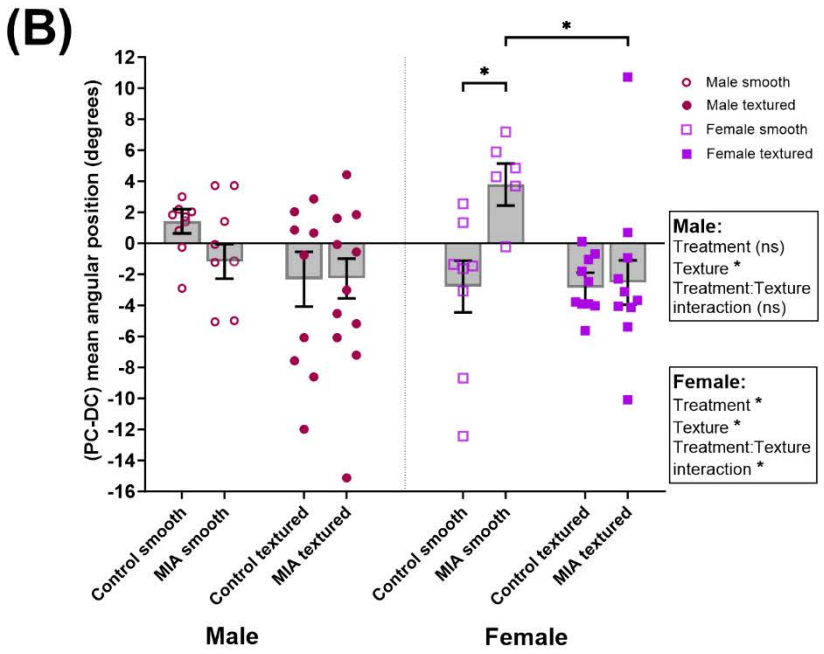
Since there was a sex effect in the above tests, the effect of treatment and object texture (or order) was then investigated in males and females separately. In female rats, there was no significant effect of treatment nor object texture on any PC whisker metrics, however, contact-related mean angular position was significantly different. Specifically, (PC-DC) mean angular position in females revealed a treatment effect, object texture effect, as well as the effect of treatment by texture interaction (treatment: $F(1, 20.750) = 5.454$, $p = 0.030$; texture: $F(1, 51.992) = 4.573$, $p = 0.037$; interaction: $F(1, 51.992) = 4.338$, $p = 0.042$, Figure 4-3B, Table 4-4). Post-hoc comparisons showed that female MIA offspring rats contacting the smooth (first) object had significantly higher (PC-DC) mean angular positions compared to female control rats contacting the same (smooth) object ($p = 0.049$) and MIA offspring rats contacting the textured (second) object ($p = 0.034$). The higher and positive (PC-DC) mean angular position of female MIA offspring rats contacting the smooth object shows the opposite of the predicted contact-related changes in their whisker angular position (i.e., defined in Table 2-1), as illustrated in Figure 4-4 where female MIA offspring rat whiskers were more symmetric and less protracted during object contact, especially when contacting the smooth (first) object (B). In contrast, MIA offspring rats contacting textured object, and control rats contacting smooth object showed the predicted patterns and pushed their whiskers more forward during contact, leading to negative values of (PC-DC) mean angular positions (Figure 4-3B, Table 4-4). These results are also supported by the example whisker traces in Figure 4-5 (corresponding video clip in Supplementary Video Clip), where the mean angular position is represented as the average line, smoothed and separated to left and right whisker sides. Figure 4-5B shows an example whisker trace from a female MIA offspring rat offspring contacting a smooth object, where mean angular position does not increase from before contact (before 0 ms) to during contact (after 0 ms).

Meanwhile, the example whisker traces of a MIA female rat offspring contacting a textured object (panel D) and control female rat offspring contacting a smooth object (panel A) both displayed increased mean angular positions during contact.

Object texture effects in female rats

There was no treatment effect, but a texture (or order) effect was found in (PC-DC) asymmetry in female rats (treatment: F (1, 20.750) = 0.086, p = 0.772; texture: F (1, 51.992) = 4.274, p = 0.044; interaction: F (1, 51.992) = 0.061, p = 0.806, Figure 4-3C, Table 4-4). While all other female rats (control rats on smooth object, control and MIA offspring rats on textured object) increased their whisker asymmetry during object contract, female MIA offspring rats contacted the smooth object more symmetrically, as indicated by positive (PC-DC) values in Figure 4-3C and a larger separation between red and blue traces in Figure 4-5B. Both female MIA offspring and female control rats contacting the smooth (first) object tended to have more symmetric whiskers (higher PC-DC values in Figure 4-3C, right), which shows the opposite of the predicted contact-related changes (in Table 2-1); however, pairwise comparisons showed no significant differences between the groups (p values > 0.05).


(A)
Mean angular position (degrees)
96
93
90
87
84
81
78
75
72
0
Male smooth
Male textured
Female smooth
Female textured
PC:
Sex *
Treatment (ns)
Texture (ns)
(PC-DC):
Sex (ns)
Treatment (ns)
Texture **
PC control
DC control
PC MIA
DC MIA
Male
Female


(B)
(PC-DC) mean angular position (degrees)
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
*
*
Male smooth
Male textured
Female smooth
Female textured
Male:
Treatment (ns)
Texture *
Treatment:Texture interaction (ns)
Female:
Treatment *
Texture *
Treatment:Texture interaction *
Control smooth
MIA smooth
Control textured
MIA textured
Male
Female

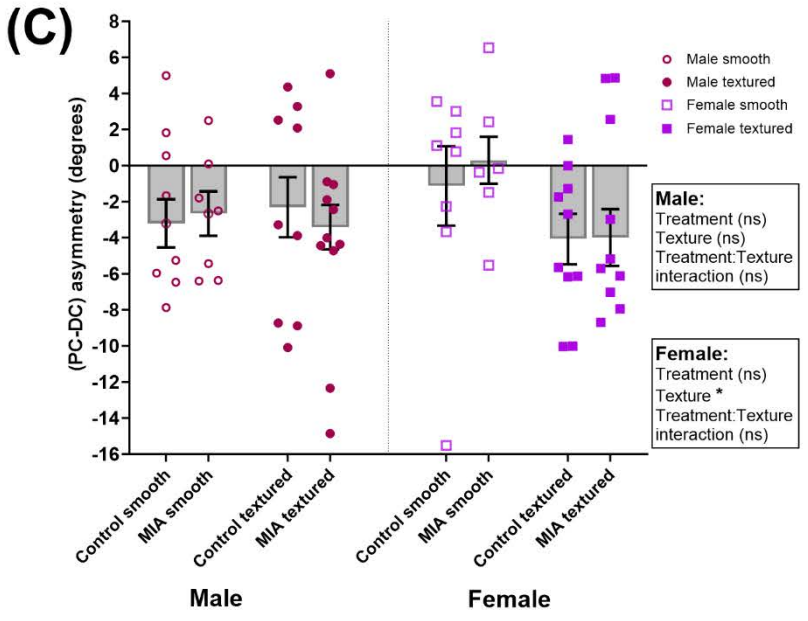

(C)
(PC-DC) asymmetry (degrees)
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
Male smooth
Male textured
Female smooth
Female textured
Male:
Treatment (ns)
Texture (ns)
Treatment:Texture interaction (ns)
Female:
Treatment (ns)
Texture *
Treatment:Texture interaction (ns)
Control smooth
MIA smooth
Control textured
MIA textured
Male
Female

Figure 4-3 **MIA offspring rat mean angular position (A and B) and asymmetry (C) are affected by treatment and object texture.** A) Significant sex effects were found in PC whisker mean angular position and object texture effects were found in (PC-DC) mean angular position. B) In males, there was a main effect of object texture in (PC-DC) mean angular position. In females, there were effects of treatment, object texture and their interaction in (PC-DC) mean angular position, while pairwise comparisons showed that MIA offspring rat smooth texture group was significantly different to control rats exploring the smooth object and MIA offspring rats exploring the textured object. C) There was a significant effect of object texture in (PC-DC) asymmetry in females, independent of treatment. Males and females were analysed together in A and separately in B and C. The bars indicate the mean values from all the clips (degrees of freedom calculated from a linear mixed-effect model), with error bars representing SEM. Data points show mean values for individual rats, indicated by open circles for male rats investigating smooth object, filled circles for male rats investigating textured object, open squares for female rats investigating smooth object, and filled squares for female rats investigating textured object. PC = pre-contact, DC = during contact, (PC-DC) = contact related changes. Asterisks mark significant values where $p \leq 0.05$ = *, $p \leq 0.01$ = ** and n.s. is not significant.

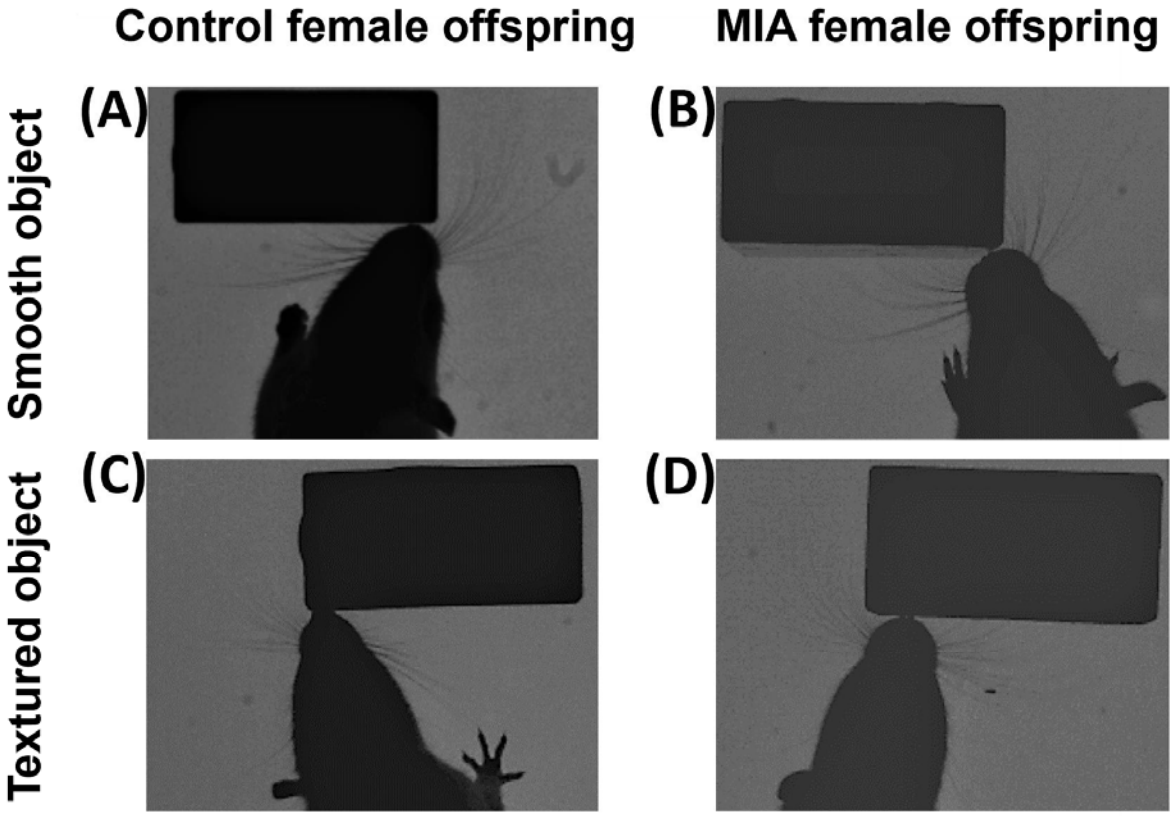


Figure 4-4 **Video stills of female offspring exploring an object.** Control female rats (A and C) show predicted object-related whisker behaviours, indicated by asymmetric positioning of the whiskers, and high whisker angular positions (e.g., more forward-reaching whiskers) following an object contact. However, the female MIA offspring rat whiskers were more symmetric and less protracted during object contact, especially when contacting the smooth (first) object (B). Video stills were selected when the whiskers were contacting the object at their maximum protraction.

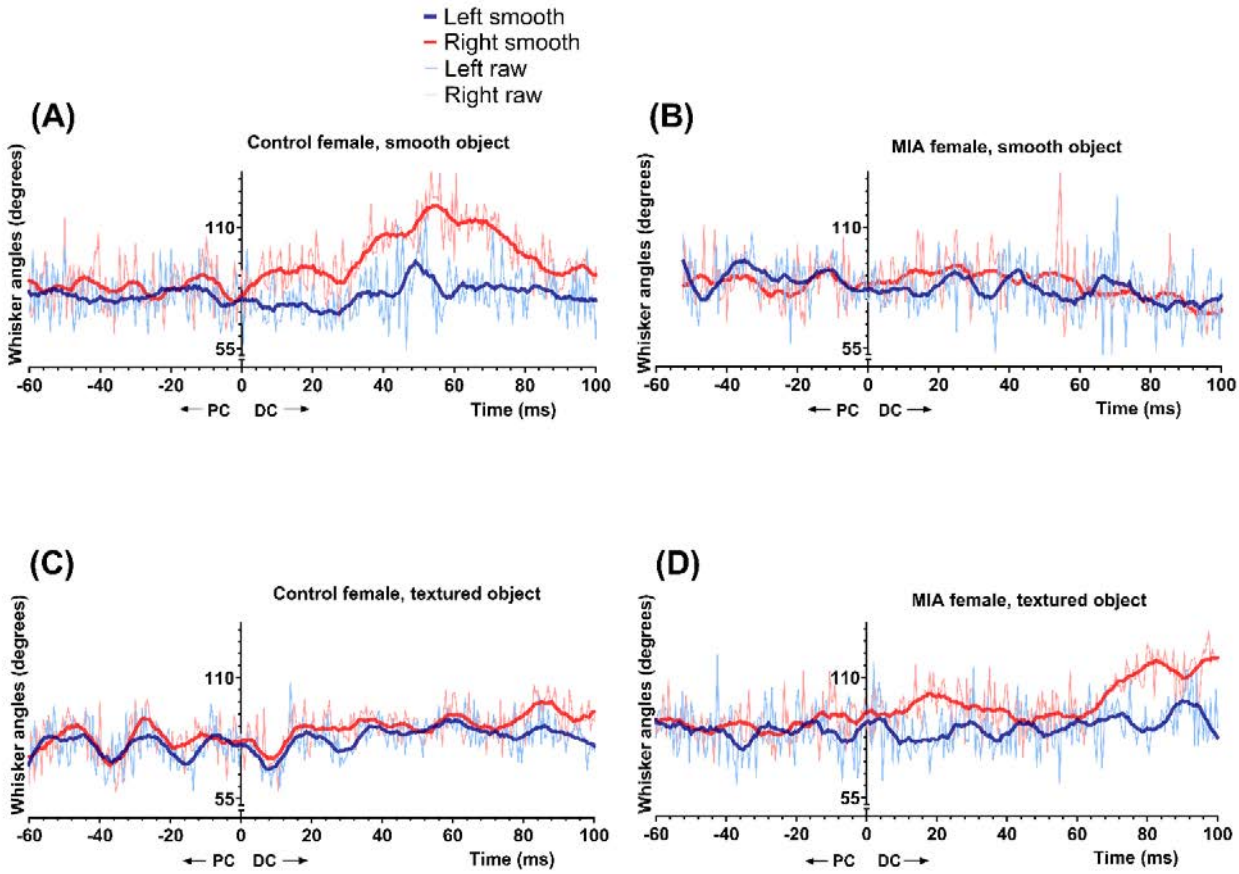


Figure 4-5 **Example whisker angle traces of female control and MIA offspring rats exploring smooth (first) and textured (second) objects.** Raw data points are shown in fine lines, and smoothed data (2nd order, 15 neighbours) are presented in thicker lines. Red colour traces are from the whiskers on the left side, and blue from the right side. 0 ms is the point of contact on the x-axis; therefore, left from the Y-axis is pre-contact (PC) and right from the Y-axis is during-contact (DC). Traces show an increase in asymmetry during contact in panels A, C and D, but not B, and an increase in mean angular position during contact in A and D but not B.

Object texture effects in male rats

There were no treatment or object texture (order) effects in male MIA offspring rats in any PC and most (PC-DC) whisker measures. There was no treatment effect in male rat (PC-DC) mean angular position, but object texture (or order) was significantly different (treatment: F (1, 19.980) = 0.307, p = 0.586; texture: F (1, 53.768) = 6.856, p = 0.012; interaction: F (1, 53.768) = 1.517, p = 0.223, Figure 4-3B, Table 4-4). While all other male rats (MIA offspring rats on smooth object, control and MIA offspring rats on textured object) pushed their whiskers more forward during object contact, male control rats on smooth object reduced their mean angular positions, as indicated by positive (PC-DC) values; however, this was not significant in further post-hoc comparisons (p values > 0.05). Nevertheless, the figures show a tendency for higher (PC-DC) mean angular position in male rats exploring smooth (first) object (Figure 4-3B male, as shown on left) compared to the textured (second) object.

Table 4-2 **Summary statistics for pre-contact data.** Linear mixed effect model and pairwise comparisons with Tukey's adjustment. Treatment, Sex and Treatment:Sex interaction columns are reported from Treatment*Sex tests, and Texture column is reported from Treatment*Texture tests. Asterisks mark significant values where $p \leq 0.05$ = *.

PC whisker parameters	Treatment effect df_1 = 1, df_2, F, p	Sex effect df_1 = 1, df_2, F, p	Treatment:Sex df_1 = 1, df_2, F, p	Texture (order) effect df_1 = 1, df_2, F, p
Amplitude (degrees)	43 2.2 0.15	43 0.74 0.39	43 1.8 0.19	100 0.29 0.59

Mean angular position (degrees)	41 2.5 0.13	41 5.2 **0.027 ***	41 1.0 0.32	110 3.6 0.059
Asymmetry (degrees)	43 1.4 0.25	43 0.75 0.39	43 0.74 0.40	110 2.0 0.16
Retraction speed (degrees/s)	38 0.098 0.76	38 0.31 0.58	38 0.63 0.43	110 0.62 0.43
Protraction speed (degrees/s)	36 0.19 0.67	36 3.3 0.076	36 0.31 0.58	110 0.88 0.35
Spread (degrees)	36 0.14 0.71	36 2.5 0.12	36 1.8 0.19	110 0.40 0.53

Table 4-3 **Summary statistics for contact-related (PC-DC) data.** Linear mixed effect model and pairwise comparisons with Tukey's adjustment. Treatment, Sex and Treatment:Sex interaction columns are reported from Treatment*Sex tests, and Texture column is reported from Treatment*Texture tests. Asterisks mark significant values where $p \leq 0.01$ = **.

(PC-DC) whisker parameters	Treatment effect df_1 = 1, df_2, F, p	Sex effect df_1 = 1, df_2, F, p	Treatment:Sex df_1 = 1, df_2, F, p	Texture (order) effect df_1 = 1, df_2, F, p
Amplitude (degrees)	38 0.0036 0.95	38 0.72 0.40	38 0.065 0.80	110 1.1 0.31
Mean angular position (degrees)	36 0.42 0.52	36 0.38 0.54	36 2.9 0.10	110 7.1 **0.0090** **
Asymmetry (degrees)	36 0.017 0.90	36 0.042 0.84	36 0.22 0.64	110 1.9 0.18
Retraction speed (degrees/s)	36 0.66 0.42	36 0.11 0.75	36 0.29 0.59	110 0.055 0.82
Protraction speed (degrees/s)	36 1.6 0.22	36 0.0012 0.97	36 0.00050 0.98	110 0.024 0.88

Spread (degrees)	41 0.059 0.81	41 0.59 0.45	41 0.0049 0.94	110 2.2 0.14

Table 4-4 **Significant whisker parameters in detail.** Here, female and male MIA rats here were analysed separately. Linear mixed effect model and pairwise comparisons with Tukey's adjustment. Asterisks mark significant values where $p \leq 0.05$ = *.

				Post-hoc			
Whisker parameters	Treatment effect $df_1 = 1$, df_2, F, p	Texture (order) effect $df_1 = 1$, df_2, F, p	Treatment: Texture (order) interaction $df_1 = 1$, df_2, F, p	MIA vs control **smooth (first)** df_1, t-ratio, p	MIA vs control **textured (second)** df_1, t-ratio, p	smooth (first) vs textured (second) **MIA** df_1, t-ratio, p	smooth (first) vs textured (second) **control** df_1, t-ratio, p
Male (PC-DC) mean angular position (degrees)	20 0.31 0.59	54 6.9 **0.012** *	54 1.5 0.22	33 -1.2 0.64	37 0.39 0.98	54 1.1 0.70	53 2.5 0.073

Female (PC-DC) mean angular position (degrees)	21 5.5 **0.030** *	52 4.6 **0.037** *	52 4.3 **0.042** *	44 2.7 **0.049** *	25 0.20 1.0	52 2.8 **0.034** *	52 0.042 1.0
Male (PC-DC) asymmetry (degrees)	17 0.17 0.69	52 0.014 0.91	52 0.22 0.64	26 0.045 1.0	41 -0.64 0.92	48 0.29 0.99	54 -0.38 0.98
Female (PC-DC) asymmetry (degrees)	21 0.086 0.77	52 4.3 **0.044** *	52 0.061 0.81	44 0.33 0.99	25 0.038 1.0	52 1.5 0.42	52 1.4 0.52

4.4 Discussion

Main findings

This study applied the established mouse whisker measurement protocol to rat offspring of the Poly I:C model to measure whisker movements before and during object exploration. Alterations in whisker movements were investigated in response to treatment, sex and object texture differences and showed that female MIA offspring rats had significant deficits in whisker movements, especially in contact-related mean angular position when contacting the smooth object. While other animals (female control rats on smooth object, male MIA offspring rats on smooth object, male and female control rats on textured object, male and female MIA offspring rats on textured object) increased their whisker mean angular

position during object contract, female MIA offspring rats reduced their mean angular positions (as supported by statistical interaction tests in Table 4-4).

Additionally, sex differences prior to contact were observed, with female rats having larger PC mean angular positions than male rats, suggesting that their whiskers were positioned more forwards prior to contact. This has been documented in mice before, where female mice had larger differences between pre-contact and during-contact values in mean angular positions than males and, because of that, were also analysed separately (Figure 2 in Grant et al., 2018). The object itself also affected whisker movements. Female rats had more symmetric whiskers on the smooth object, compared to the textured object, while male rats did not increase their whisker mean angular positions during contact with the smooth object, as they did on the textured object. These results suggest that whisker tracking is sensitive enough to identify both sex differences and treatment effects caused by the Poly I:C treatment.

Poly I:C treatment effects

Firstly, when male and female rats were grouped, no effect of Poly I:C treatment was seen on any of the PC or (PC-DC) whisker metrics (Figure 4-2). In a study with a similar experimental design with respect to MIA induction, housing, and caging systems, Potter et al. (2023) also found no effect of Poly I:C treatment in adolescent or adult male or female rats in a NOR task used to assess visual learning. Similarly, in male and female adolescent or female adult rats (adult males were not tested) there was no treatment effect in an elevated plus maze task used for measuring anxiety-related behaviour (Potter et al., 2023). Therefore, the behavioural deficits in these adult animals are recognised as only subtle, but they can be detected with a quantitative test, such as the whisker measurement protocol adopted here.

Subsequently, treatment effects were observed in contact-related (PC-DC) whisker measures when the data set was separated into males (n = 25) and females (n = 25). Specifically, female MIA offspring rats were found not to increase

their mean angular position during contact with the smooth (first) object, implying they did not engage in the predicted contact-related behaviours (e.g. defined in Table 2-1). In contrast, control rats increased their whisker position (positioned them more forward) during contact, demonstrating the predicted contact-related behaviour. This more forward whisker positioning during object contact is associated with focussing of attention (Arkley et al., 2014; Mitchinson et al., 2007), which was absent in the MIA female offspring rats contacting the smooth object, and may, therefore, suggest attentional deficits in MIA female offspring. Potter et al. (2023) also found multiple deficits in attention and problem solving in female MIA offspring rats as tested by the attentional set-shifting task (males were not tested) and significantly more errors were made in the radial arm maze task, suggesting that their spatial working memory is impacted by the Poly I:C treatment (males were not tested; Potter et al., 2023). Lins et al. (2019) found an impairment in an odd (dissimilar) object recognition task which relies on the ventral visual stream. By measuring the exploration time in an arena with three identical and one odd object, they found that both male and female MIA offspring rats explored the odd object for less time than the control group. Different tests by Lins et al. (2019) showed that both visual object recognition memory in males and females, as well as cross-modal (visual and tactile) object recognition memory in males were affected by Poly I:C treatment, while tactile-only object recognition was unaffected. However, their tactile object recognition task only measures the duration of exploration (timings), rather than the highly detailed and quantitative measurements of whisker movements used in this study; this might account for the different results. To fully understand the potential of whisker tracking as a measure of attention, it would be beneficial to directly compare it to a gold-standard task that measures attentional deficits, such as the five-choice serial-reaction time task (Robbins, 2002, Young et al., 2009a, Lustig et al., 2013).

Sex effects

While sex as a biological factor is important to consider in any research, it is especially relevant when modelling NDDs, which affect males and females

differently (Kokras and Dalla, 2014). Combining data from males and females, rather than testing each sex separately can mean loss of granularity, as natural biological sex-dependent differences can mask the effect of treatment. Hence, it is important to include both sexes and assess the effects of treatment on them separately, as was done here (sex-based reporting, Yoon et al., 2014, Miller et al., 2017).

A general sex effect in PC mean angular position was found, where female rats had larger (more forward) whisker positions before contact, compared to male rats. When analyses of males and females were conducted separately, more object texture differences were found in female than in male rats. Potter et al. (2023) also found several sex-specific effects in MIA offspring rats, mainly in pups, where female MIA offspring gained less weight in the early postnatal period, compared to males. Female MIA offspring pup solicitation behaviours (ultrasonic vocalisations) and grooming were also affected by the Poly I:C treatment up to PD14. However, Potter et al. (2023) did not find an effect of sex in either adolescence or adulthood on the NOR task used to assess visual learning. In Lins et al. (2019), visual object recognition memory and cross-modal object recognition memory were also not directly affected by sex. Tracking whisker movements quantitatively may provide a more sensitive description of sex differences, since a significant difference in mean angular position before object contact was observed, a parameter that the previously mentioned object recognition tasks did not measure. Several differences in treatment related deficits have been observed between male and female rats in Lins et al., 2018 and 2019. While oddity preference and visual object recognition memory were affected in both males and females (Lins et al., 2018 and 2019), the cross-modal object recognition memory was only impaired in males (Lins et al., 2018). It is not wholly clear why in their study male rats were affected more, compared to this study and Potter et al. (2023) studies where female rats displayed more effects of Poly I:C treatment. However, the dose of Poly(I:C), its gestational timing and the background strain of the rat will all contribute towards this variability between studies.

Object texture or sequential order effects

To really understand if the object differences seen in this study were caused by the order of object presentation or their texture, the order should have been randomised and any similar studies in the future should do so. As it was not done this time, the texture effect referred to in this section may actually be an order effect.

For the first time, significant differences between treatments were observed in whisker movements during a sequential object task. Specifically, when data from male and female rats were separated, significant differences were identified between female MIA offspring rats contacting smooth and textured objects. Female MIA offspring rats contacting the smooth object did not increase the mean angular position of their whiskers during contact, whereas female MIA offspring rats contacting the textured object and control rats touching the smooth object all did increase their mean angular position during contact. Moreover, both MIA and control female offspring rats contacting the smooth object tended to engage in contact-induced asymmetry less than male rats (Figure 4-4, Figure 4-5). Increases in forward whisker mean angular positions and asymmetry during contact are most typically observed (Berg and Kleinfeld 2003, Grant et al., 2009; Mitchinson et al., 2007, 2011). The absence of these behaviours on the smooth object may suggest an abnormal behaviour and lack of focussing of the whiskers on to this particular object (Arkley et al., 2014; Mitchinson et al., 2007). Therefore, female MIA rat offspring may have attentional deficits, that especially manifest during whisker exploration of this first object presented to them.

The sequential object exploration task is related to memory and, considering that the objects were very similar and only differed in colour and texture (Figure 4-1), the novelty of the second object is not especially pronounced. However, for the first time, a treatment effect was detected in whisker-related measures in this task. Specifically, whisker movement deficits were present in contacts with the first object and not the second object. An identical sequential object exploration task was used by Landreth et al. (2021) in a sub-chronic PCP rat model of

schizophrenia and did not reveal any differences between the smooth and textured objects. Grant et al. (2018) also used a sequential object exploration task with two different objects in a mouse model of Alzheimer's disease, and while all mice showed differences between contacting the first and the second object, with smaller whisker amplitudes during contact with the second object, there were no treatment differences, but a significant sex effect was present. Treatment effects were found in this study during whisker contacts with the smooth (first) object, but not the textured (second) object. Therefore, the number, type (or texture) and novelty of objects used in exploration tasks can be important when looking for MIA treatment effects.

The sequential object exploration task is probably the most similar to a NOR task, one of the gold-standard behavioural tests, suggested to be translatable to human symptoms (Young et al., 2009b). Only NOR and social tasks rely on innate behaviour, while other cognitive tasks require training and motivation by either food or aversion. It has been shown several times (Arkley et al., 2014; Grant et al., 2016, 2018; Simanaviciute et al., 2022) that whisker tracking during novel object exploration (NOE), as opposed to during a recognition task, can also assess attention, but without any training or habituation. The work presented here shows that the sequential object exploration task can also be used for the same purpose, and that the addition of a second object, like a NOE task, allows assessment of attention in more detail depending on the choice of objects. It might be beneficial to combine whisker tracking with the classic NOR task, to study the object contact in more detail and to incorporate the memory aspect when assessing these measures with novel and familiar objects. Indeed, whisker movements can be measured during most tasks and can offer a refinement to animal testing by providing highly quantitative data, without the need for training, reward, or habituation.

4.5 Method development

The whisker measurement protocol is usually carried out in mice. In this chapter, the protocol was tested and for use in rats, including both males and females and

provides evidence that it is possible to adapt the set up for rats - the second largest group of animals used in laboratory experiments throughout the world. This is a great advantage for neuroscience researchers that prefer using rats, especially for cognitive and aging studies.

An additional component to the usual experimental procedures is the addition of a sequential object task, so two objects were used rather than the usual one. This revealed significant effects in MIA female offspring on the smooth object, but not the textured one. Object selection and object comparisons might be a useful tool in future studies. However, since the objects are very similar, only differing in texture, habituation to the object and the arena might also be a factor that is worth exploring too. I will investigate arena habituation in my next chapter, Chapter 5.

For the first time, following my method development and considerations in the previous chapter (Chapter 3), I also did not include any qualitative scoring - looking to the quantitative measurements of mean angular position, amplitude, asymmetry and spread, instead of scores of whisking, spread reduction and CIA. This has significantly reduced the time associated with processing and scoring videos and removed the main manual element of the process. Therefore, replacing with qualitative scoring with the quantitative metrics will be continued into Chapter 5.

The next step to developing this method further is to incorporate a second camera to allow the validation of whisker behaviour against general locomotor behaviour throughout the trial.

4.6 Conclusion

By testing the protocol in rats and including male and female animals, this study further contributed to my first PhD objective which is the standardisation of this method. Female offspring of MIA-induced rat dams revealed deficits in contact-related whisker movements, suggesting an attentional deficit caused by the Poly I:C treatment. Whisker tracking is suggested as a useful tool to assess attentional deficits in models of NDDs. For the first time, treatment effects were revealed by a

sequential object exploration task, which allows to also study the effect of object type or order and contributed to the third PhD aim, which is integration with other behavioural tasks. This is especially important when deficits in whisker movements occur, but only when interacting with one type of object and not another. Additionally, the importance of including both male and female animals and evaluating their neurological symptoms separately is emphasised, as treatment effects can be masked by innate sex differences. Overall, measuring whisker movements is demonstrated here as a quick, non-invasive, quantitative tool that is sensitive enough to identify treatment effects and sex differences during object exploration in an adult offspring model of NDDs caused by MIA.

4.7 Supplementary materials

Cross-fostering

Table 4-5 **Summary statistics for cross-fostering effects on pre-contact data.** Linear mixed effect model and pairwise comparisons with Tukey's adjustment. Asterisks mark significant values where $p \leq 0.05$ = * and n.s. is not significant. All post-hoc tests were not significant.

PC whisker parameters	Treatment effect $df_1 = 1$, df_2, F, p	Cross-fostering effect $df_1 = 1$, df_2, F, p	Treatment: Cross-fostering interaction $df_1 = 1$, df_2, F, p
Amplitude (degrees)	44 1.5 0.23	44 4.2 **0.047** * - all n.s. in post-hoc tests	44 0.86 0.36
Mean angular position (degrees)	43 1.4 0.24	43 0.028 0.87	43 0.21 0.65
Asymmetry (degrees)	44 1.1 0.30	44 0.012 0.91	44 0.16 0.69
Retraction speed (degrees/s)	41 0.0079 0.93	41 0.55 0.46	41 1.8 0.19

Protraction speed (degrees/s)	41 0.0046 0.95	41 0.091 0.77	41 0.29 0.59
Spread (degrees)	41 0.0027 0.96	41 2.0 0.17	41 0.35 0.56

Table 4-6 **Summary statistics for cross-fostering effects on contact-related (PC-DC) data.** Linear mixed effect model and pairwise comparisons with Tukey's adjustment. All post-hoc tests were not significant.

(PC-DC) whisker parameters	Treatment effect $df_1 = 1$, df_2, F, p	Cross-fostering effect $df_1 = 1$, df_2, F, p	Treatment: Cross-fostering interaction $df_1 = 1$, df_2, F, p
Amplitude (degrees)	41 0.033 0.86	41 1.5 0.23	41 0.22 0.64
Mean angular position (degrees)	40 0.43 0.52	40 0.29 0.59	40 0.0047 0.95
Asymmetry (degrees)	40 0.0047 0.95	40 0.35 0.56	40 0.019 0.89
Retraction speed (degrees/s)	40 0.38 0.54	40 0.16 0.670	40 1.8 0.019
Protraction speed (degrees/s)	40 1.6 0.22	40 1.4 0.25	40 1.2 0.28
Spread (degrees)	42 0.38 0.54	42 3.9 0.056	42 0.0036 0.95

Whisker movements

Table 4-7 **Summary statistics for Treatment*Texture effects.** Linear mixed effect model and pairwise comparisons with Tukey's adjustment. Texture effect is reported in Tables 3-3 and 3-4. Males and females analysed together.

Whisker parameters	Treatment effect df_1 = 1, df_2, F, p	Treatment:Texture effect df_1 = 1, df_2, F, p
PC amplitude (degrees)	46 2.5 0.13	100 3.0 0.090
PC mean angular position (degrees)	45 1.4 0.24	110 0.070 0.79
PC asymmetry (degrees)	44 1.3 0.26	110 0.20 0.65
PC retraction speed (degrees/s)	39 0.14 0.71	110 0.021 0.89
PC protraction speed (degrees/s)	38 0.010 0.92	110 1.4 0.24

PC spread (degrees)	40 0.0035 0.95	110 0.015 0.90
(PC-DC) amplitude (degrees)	40 0.0042 0.95	110 0.35 0.56
(PC-DC) mean angular position (degrees)	38 0.82 0.37	110 0.047 0.83
(PC-DC) asymmetry (degrees)	38 0.0044 0.95	110 0.058 0.81
(PC-DC) retraction speed (degrees/s)	38 0.83 0.37	110 0.080 0.78
(PC-DC) protraction speed (degrees/s)	38 2.3 0.14	110 1.4 0.24
(PC-DC) spread (degrees)	43 0.32 0.58	110 1.9 0.17

CHAPTER 5 HOMOZYGOUS REELER MICE REVEAL WHISKER MOVEMENT DEFICITS PRE-CONTACT WITH A NOVEL OBJECT AND DURING AN OPEN FIELD HABITUATION TASK

Chapter summary

Reeler mice have been suggested as a model for several disorders, including the developmental disruption of cortical layers, lissencephaly type 2 and perhaps even epilepsy. Homozygous reeler mice were chosen here since they have a complex behavioural phenotype and few studies have revealed robust behavioural and cognitive deficits in these mice, despite their neuroanatomical disruptions. Using the standard whisker protocol, only one whisker measure – pre-contact spread - was found to be affected by genotype. Moreover, an infrared 30 fps camera was paired with a high-speed camera to record the whole object exploration experiment, rather than just the small periods of exploration that are usually captured. This showed that reeler mice spent the same length of time around the object as wildtype mice. Further testing revealed that previous exposure to the arena might influence whisker measures obtained using the standard protocol. Additional testing was performed where whisker movements were filmed with the high-speed camera in an open field environment and during a further habituation period of five sessions. Whisker movements were compared in the open field as well as in the first and fifth habituation sessions. Habituation affected locomotion speed, whisker amplitude, mean angular position and spread. Then, high-speed video clips captured during novel object recognition task were revisited to measure the nose to object distance. Statistically, the distance from object was not different, although reeler mice were generally further from the object. I posit distance from the object might have influenced the number of clips that were included in the original analysis. Whisker movements remain a powerful behavioural measurement tool; however, the usual protocol might not suit all mouse models, especially if their impairment does not allow for a close contact with the object.

5.1 Introduction

Reeler mice (Falconer, 1951) have a mutation in the reelin gene responsible to produce the reelin protein, which is involved in cerebral cortex development and the formation of cortical layers (D'Arcangelo et al., 1995). Reelin gene mutation results in mice with visible motor impairment (ataxia, dystonia) and cerebral cortex abnormalities, specifically the absence of layers in the somatosensory cortex, where neurons would normally stack up into 6 layers (D'Arcangelo et al., 1995; see Figure 1-2 of this thesis) and differences in vasculature architecture (Inoue, 1990). Hence, homozygous reeler mice are used as a model for the developmental disruption of cortical layers. They have also been proposed as a model of lissencephaly type 2, a rare human developmental disease characterised by abnormal brain pathology and congenital muscular dystrophy (Lossi et al., 2019). Homozygous reeler mice have also been found to display epileptic seizures during recovery from isoflurane anaesthesia, while their wildtype counterparts did not (Kopjas et al., 2006); however, a reeler mouse model of epilepsy is still in its development (Lossi et al., 2019).

Up until recently, it has been thought that layers have an important function in how neurons are connected and work in the brain. Therefore, it was surprising to see that reeler mice have only a slight cognitive impairment in spatial memory and executive function (Goldowitz and Koch, 1986). New studies have since challenged the idea of layers being a mandatory component of a normally functioning brain, mostly from studying reeler mice, where brain plasticity has enabled reeler mouse neurons to be almost as functional and connective as those of wildtype mice, despite their distorted brain structure (Guy and Staiger, 2017, see Figure 1 of the paper referenced for how cortical layers of a control mouse look like next to the absence of lamination in a reeler mouse). For example, the topological whisker representations in the barrel field were found to be preserved with individual whisker representations like that of wildtype mice (Guy et al., 2015). Additionally, the basic visual abilities in reeler mice are unaffected, but the more complex perception tasks, such as orientation discrimination, are compromised; interestingly, reelin deficiency seems to enhance the juvenile visual plasticity

levels into the late adulthood age (Pielecka-Fortuna et al., 2015). In terms of motor disturbances, homozygous reeler mice not only exhibit ataxic gait but also have a deficit in forepaw dexterity, including paw guiding and grasping movements, as well as an increase in the threshold of currents required to evoke forelimb movements from an intracortical microstimulation (Nishibe et al., 2018).

Reeler mice are of particular interest in my thesis because they lack layers in the barrel cortex (does not have the standard structure as in Figure 1-2 C), which contains the structural representation of whiskers. They also have reduced inputs to the motor cortex, increased inputs to the contralateral barrel cortex, as well as a larger corpus callosum from more callosal projection neurons (Hafner et al., 2021) which may also impact whisker movements. For example, I might expect that the increased projections to the corpus callosum may affect bilateral whisker behaviours, such as contact-induced asymmetry. Reeler mice have major motor disturbances, including ataxic gait and extrapyramidal tics; however, most behavioural studies in reeler mice do not reveal significant cognitive disturbances (Guy and Staiger, 2017).

The tactile sensory function of reeler mice has not been extensively studied before, although I have previously found differences between heterozygous reeler and wildtype male mice in the whisker mean angular position at 7-9 months (Simanaviciute et al., 2020). However, a more extensive study is needed to truly understand whether whisking in reeler mice is different between male and female mice, as well as whether there are any differences in homozygous reeler mice. The aim of Chapter 5 is to validate the whisker tracking protocol against common measures of exploration, which addresses my third PhD objective – including time spent in the arena at the walls, centre and object. Homozygous reeler mice were chosen for investigation here, since they have a complex behavioural phenotype, and few findings have shown strong behavioural and cognitive deficits, despite their neuroanatomical disruptions (Guy and Staiger, 2017). In this chapter, I first investigate whisker movements before and during novel object exploration as well as in an open field to study the motor, sensory and attentional phenotype of reeler mice. I then further develop the protocol by introducing an additional overhead

camera to find out if such integration is feasible and advantageous. Habituation to the arena will then be investigated in additional open field sessions, in combination with the current whisker tracking protocol, to understand if the method can be applied in rodent disease models where the approach to an object is affected.

5.2 Materials and methods

Animals

Reeler mice in this study were genetically designed to be able to visualise three neuronal populations in their brain - vasoactive intestinal peptide (VIP), somatostatin (SOM) and vasopressin (VP) - for parallel brain anatomy studies. The behaviour of reeler mice from different lines was not expected to be different, but it was tested before combining all reeler mice together. The reeler line (B6C3Fe a/a-Relnrl/J, The Jackson Laboratory) was crossed with the VIP-Cre, SOM-Cre and PV-Cre lines to breed VIP-Cre/reeler, SOM-Cre/reeler and PV-Cre/reeler mice heterozygous for reelin mutation. These animals were then crossed to generate VIP-Cre/reeler, SOM-Cre/reeler and PV-Cre/reeler mice, all homozygous for reelin knockout. To visualize the population of VIP, SOM and PV cells, the VIP-Cre/reeler, SOM-Cre/reeler and PV-Cre/reeler line were crossed with the Ai9 tdTomato reporter line (B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J, The Jackson Laboratory) to achieve tdTomato expression in VIP, SOM and PV neurons. Wildtype littermates were used for comparison in the experiments. Mice were housed in groups in standard cages in a reversed 12-hour light/dark cycle (lights on from 7 pm to 7 am) and with ad libitum access to food and water. All experimental procedures were performed in accordance with German laws on animal research. The experimental protocol was approved by the local ethics committee at the University of Göttingen and at Manchester Metropolitan University (reference no. 2019-11562-7372).

Due to the low survival rate in reeler mice, it is difficult to produce mice in specific age groups. Mice in these experiments included both males and females aged between 2 and 8 months of age. 25 animals undertook the novel object exploration and open field experiments. However, in the open field and object exploration task,

6 mice were previously exposed to the test arena for 5 open field habituation sessions (10 minutes each) and 1 texture recognition task (10 minutes) where mice explored two objects different from the object used in this study. Even though the final exposure to arena was less than 24 hours before the main experiment, they were found not to behave any differently from naïve mice in the open field, so they were included in the open field tasks. After clip selection (see criteria below), 22 mice were included in the open field study, which included naïve and previously exposed mice (Table 5-1, Open field).

Table 5-1 **Animal numbers** for each task, including the breakdown for genotype, sex, line, age and previous exposure to the arena.

Open field				
Genotype	12 reeler	10 wildtype		
Sex	13 male	9 female		
Line	6 VIP	6 SOM	10 VP	
Age	8 mice	4 mice	6 mice	4 mice
	2 months	3 months	4.5 months	8 months
Previous exposure	18 naïve	4 previously exposed		
Novel object exploration				
Genotype	9 reeler	9 wildtype		
Sex	12 male	6 female		
Line	3 VIP	5 SOM	10 PV	
Age	8 mice	4 mice	5 mice	1 mouse
	2 months	3 months	4.5 months	8 months
Previous exposure	18 naïve	0 previously exposed		
1st Habituation				
Genotype	10 reeler	11 wildtype		
5th Habituation				
Genotype	9 reeler	8 wildtype		

Unlike the open field, the novel object exploration study brought out significant differences in whisker movements of mice that were previously exposed to the test arena, compared to those of naïve mice (data presented in 5.3 Results and 5.4 Discussion). Hence, for further analysis, the 6 previously exposed mice were removed from the object exploration study, leaving 18 mice for inclusion following clip selection (Table 5-1, Novel object exploration). The habituation study started off with 24 mice and after clip selection ended up with 21 mice in the 1st habituation and 17 mice in the 5th habituation. The breakdown of genotype, sex, line, age and previous exposure status can be found in Table 5-1.

Experimental procedures

A Pyrex glass bottle stopper (Figure 5-2A) was placed inside the arena to facilitate exploratory behaviour for 5-10 mins and then taken away for another 5-10 minutes of open field exploration. For this part of the study, all mice were filmed on the same day. For all other methods which remain unchanged across the experimental Chapters, please refer to Chapter 2 General methods.

An additional overhead infrared camera (DVcam) was introduced to record the whole session of the exploratory task at 30 frames per second with a resolution of 1920 × 1080 pixels. This was the first time another camera was introduced alongside high-speed video filming. For part two of this study, the same mice were placed in the same arena for additional habituation trials. This time, mice were tested on different days from part one of the study. Up to 6 mice per day were habituated 5 times for 10 minutes each. The 1st and the 5th habituation sessions were captured using the same videography techniques as the first part of the study. Both parts of the study are illustrated in Figure 5-1 with references to animal sample sizes at each stage.

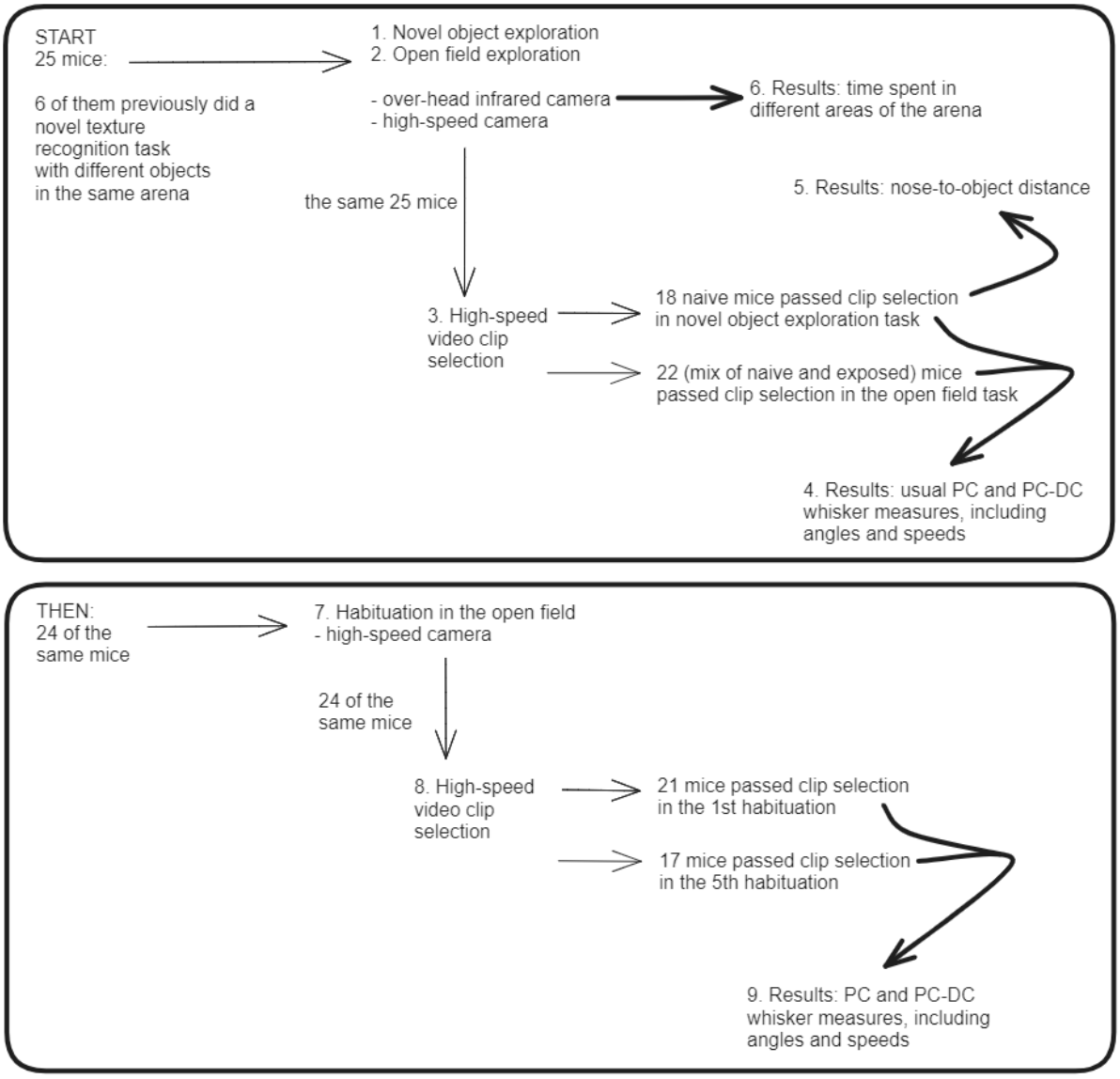


Figure 5-1 A scheme showing the sequence of experiments in Chapter 5 and animal numbers at each stage.

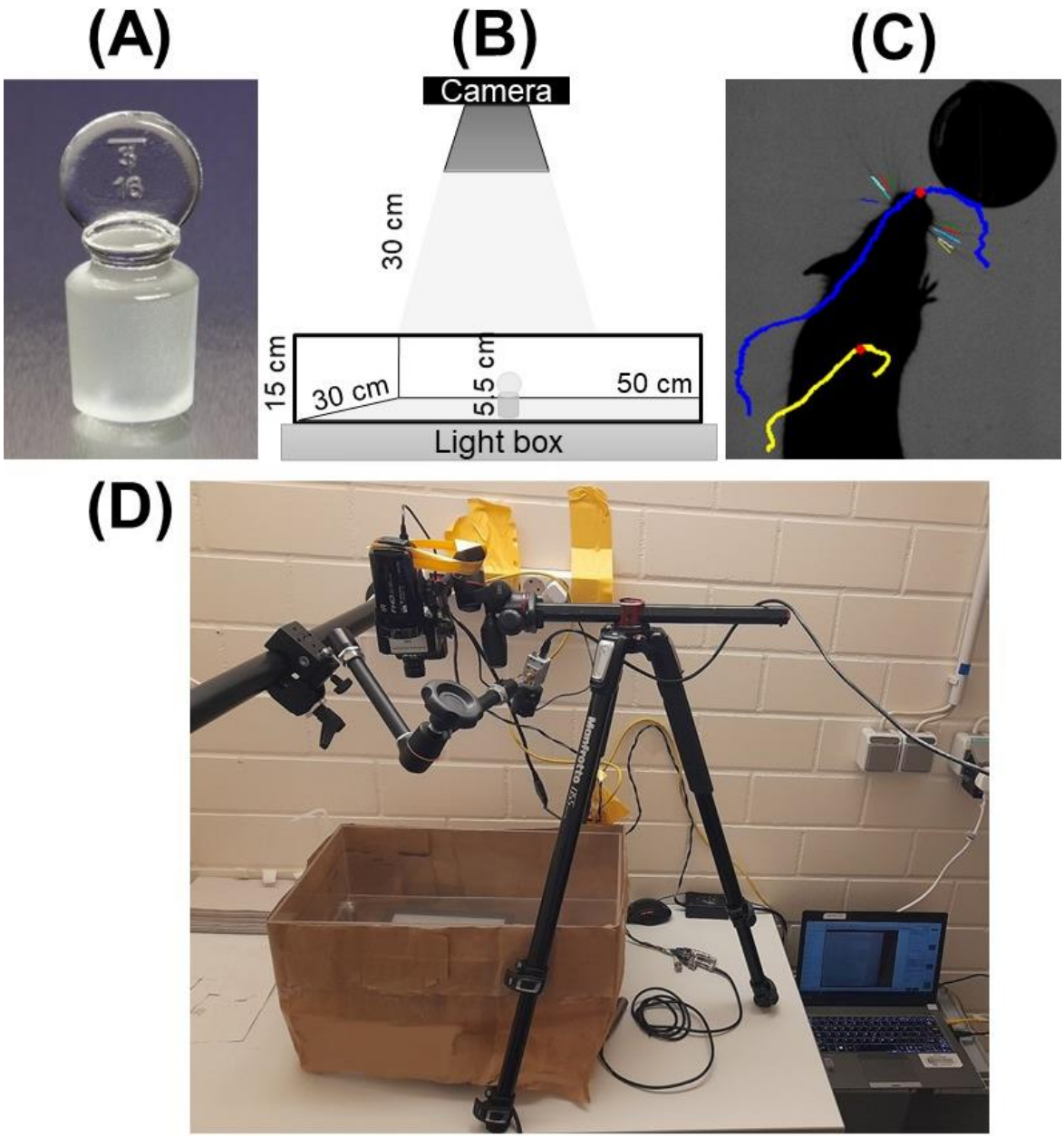


Figure 5-2 **Experimental set up.** Panel A shows the glass bottle stopper object used in the novel object exploration task; Panel B illustrates the filming set-up, the object size and location in relation to the Perspex box and the distance between the arena and high-speed video camera. The field of view in light grey corresponds to the video stills in Panel C showing an example high-speed video clip. ARTv2 LocoWhisk software was used to automatically locate the mouse centroid (red point, yellow line), nose tip (red point, blue line) and whiskers (coloured lines), and detects them on a frame-by-frame basis. Panel D shows the set up with both the highspeed and overhead camera placement.

High-speed video analysis

1-9 clips per mouse were included in data analysis; a total of 110 open field clips and 73 (without previously exposed mice) object exploration clips were included in the analysis. In the habituation study, 1st habituation had 189 video clips, and the 5th habituation included 106 clips. For nose to object analysis 152 clips were included.

Nose to object distance and general behaviour

Novel object exploration clips were revisited to investigate if reeler mice got as close to the object as wildtype mice. The tape measure tool in Tracker (6.1.5) was used to manually measure the shortest distance from the tip of the nose to the object, once per video clip, when the mouse was the closest to the object. This allowed the assessment of video clips that normally would be removed due to the clip selection criteria for the DC part, such as when whiskers were not contacting the object, or the mouse did not orient and further explore the object. Data points were averaged per mouse for analysis.

For general behaviour, mice were manually timed using BORIS software (v.8.20, Friard and Gamba, 2016) in the overhead infrared video clips. Mice were recorded throughout the whole duration of the novel object exploration and open field trial. Durations were measured for the time spent around the arena walls, in the middle of the arena and around the object, giving one data point for each mouse per arena position.

Histology and cell count

Two mice (1 reeler 5-month-old female and 1 wildtype 6.5-month-old male) were euthanised via an approved schedule 1 procedure and perfused. The brain was removed and then sectioned on a vibratome (VT 1200 S, Leica) into 100 µm thick coronal sections rostral and caudal to the barrel cortex (like Hafner et al., 2021). Free-floating slices were stained for VGAT (1:1500), VGLUT1 (1:1000) and DAPI following a standard immunohistochemistry protocol, as per the antibody

manufacturer recommendations. VGAT (Synaptic Systems, Germany) is a monoclonal antibody which identifies glycine and GABAergic connections, while VGLUT1 (Synaptic Systems, Germany) is a polyclonal antibody used to identify glucose transporter proteins. Sections were also stained with DAPI, diluted 1:1000 in TRIS buffer solution (TB, consisting of 20 mM Tris, pH 7.2, 150 mM NaCL), which stains cell nuclei blue. After several washes in TB, sections were mounted in Aqua-Poly-Mount (Polysciences) and coverslipped.

Images of the slides were acquired as z-stacks using Leica fluorescence Thunder Imager with a x63 oil immersion objective. The Mouse Brain Atlas (Paxinos and Franklin, 2013) was used to identify the regions of interest: corpus callosum (CC), motor cortex (M1) and primary somatosensory cortex barrel field (S1BF). 32 images were captured from wildtype mice and 28 images from reeler mice. On review of the histology, the VGAT and VGLUT1 stains had not penetrated the whole slide, therefore these were not included in further analyses and cell counts were conducted on the DAPI stained nuclei only.

Statistical analyses

For all whisker and locomotion measurements, each variable was compared between wildtype and transgenic mice. Checks for differences between sex, age, line and previous exposure and graphs for object exploration and habituation studies were produced in GraphPad Prism 9. All other statistical tests were run in R Studio. A Linear Mixed-Effects Model (LMEM) was to analyse the effect genotype, line, age and sex on all PC and PC-DC whisker variables. The model computed F tests on the fixed effects and provided p-values using a type III ANOVA.

The nose distance and neuroanatomical data were analysed using unpaired t-tests and graphed in Microsoft Excel (version 2308). The overhead video clips were analysed as percentage of time spent in the areas of arena and statistically evaluated using Pearson's Chi-squared test.

5.3 Results

Significant differences were found between the previously exposed and naïve animals (unpaired t-tests, GraphPad Prism) in PC asymmetry (df = 22, t = 2.168, p = 0.0413), PC spread (df = 22, t = 2.113, p = 0.0462) and PC-DC protraction speed (df = 22, t = 3.118, p = 0.0050). Therefore, the 6 previously exposed mice were removed from further novel object exploration analyses and the results in this chapter only include naïve mice. Data from previously exposed mice are, however, included in the figures (Figure 5-3 and Figure 5-4) separately from naïve mice but were not analysed due to being such a small sample size. Main results from LMEM models are reported in Table 5-2.

Pre-contact (PC) quantitative whisker and locomotion movements

A genotype effect was found in PC whisker spread when testing Genotype by Sex (genotype: F (1, 12.057) = 5.4403, p = 0.03781; sex: F (1, 12.057) = 0.2001, p = 0.66254; interaction F (1, 12.057) = 2.5175, p = 0.13845), Figure 5-3, sex effects not shown, Table 5-3). However, pairwise comparisons showed no significant differences in post-hoc tests (p values > 0.05). The same PC whisker spread measure was not significantly different when tested in Genotype by Age (genotype: F (1, 9.2176) = 1.1135, p = 0.3182; age: F (3, 9.1696) = 0.5064, p = 0.6873; interaction F (2, 9.3618) = 0.0885, p = 0.9160), Table 5-3) nor in Genotype by Line (genotype: F (1, 9.4032) = 2.9525, p = 0.1184; line: F (2, 9.5170) = 2.1930, p = 0.1647; interaction F (2, 9.5170) = 0.0677, p = 0.9350).

There were no genotype nor sex differences in other PC metrics, including PC amplitude (genotype: F (1, 11.585) = 0.5489, sex: F (1, 11.585) = 0.1817; interaction F (1, 11.585) = 0.3880), PC mean angular position (genotype: F (1, 10.54) = 1.9549; sex: F (1, 10.54) = 3.3027; interaction F (1, 10.54) = 4.0765), PC asymmetry (genotype: F (1, 10.54) = 1.7344; sex: F (1, 10.54) = 2.3691; interaction F (1, 10.54) = 1.4471, all p > 0.05, Figure 5-3, Table 5-3), PC locomotion speed (genotype: F (1, 11.427) = 2.7201, sex: F (1, 11.427) = 0.0806; interaction F (1, 11.427) = 0.6950), PC retraction speed (genotype: F (1, 10.54) = 0.1981; sex: F (1, 10.54) = 0.5499; interaction: F (1, 10.54) = 1.3600) and PC

protraction speed (genotype: F (1, 10.54) = 0.2877; sex: F (1, 10.54) = 0.8972; interaction: F (1, 10.54) = 0.8442, all $p > 0.05$, Figure 5-4, Table 5-3).

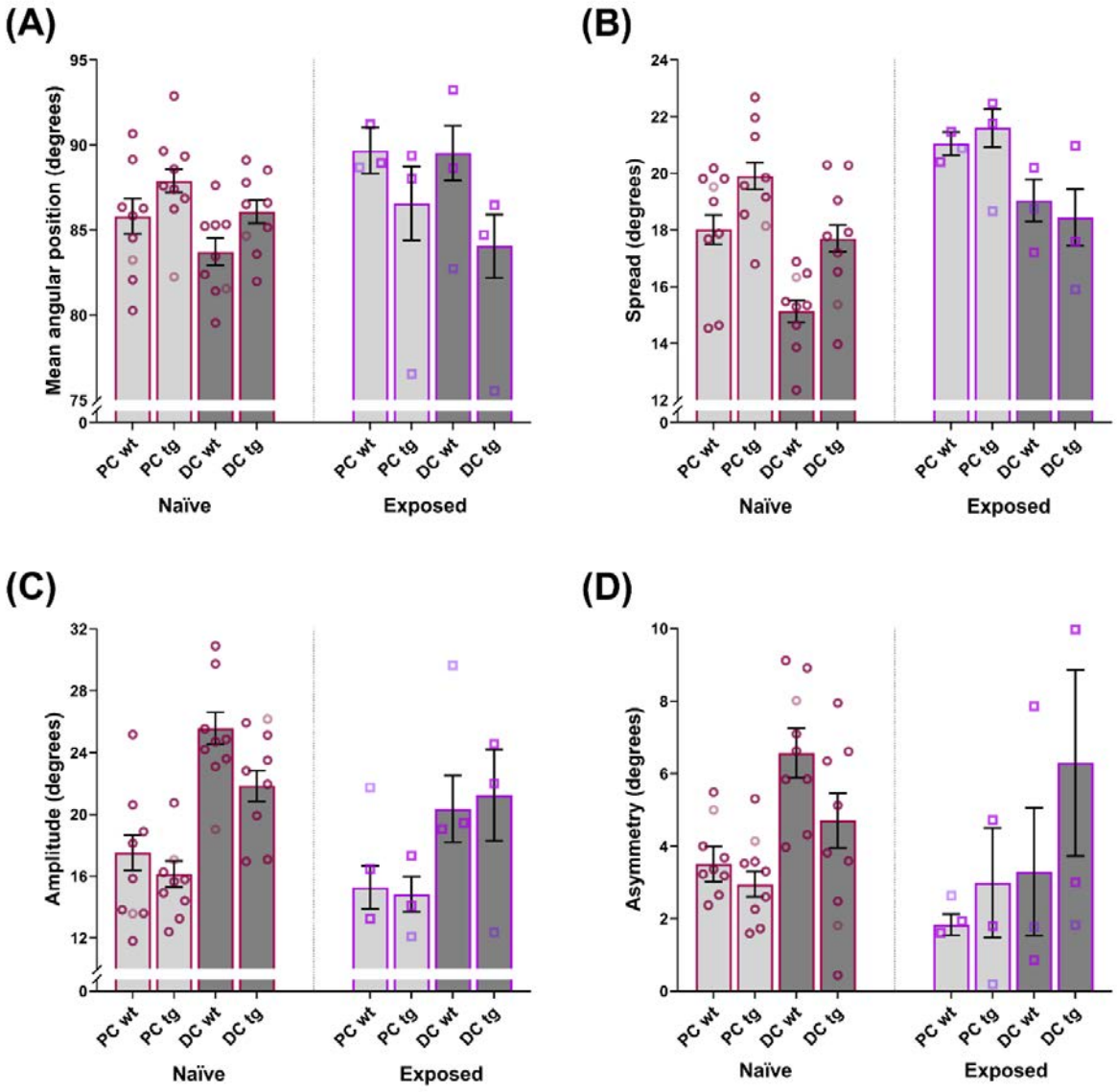


Figure 5-3 **Angular whisker movement measurements: mean angular position, spread, amplitude and asymmetry.** Note the increase in amplitude (C), asymmetry (D) and a decrease in spread (B) from PC to DC part of the video clips representing the robust contact-related changes in naïve mice. The bars indicate the mean values from all the clips (degrees of freedom calculated from a linear mixed-effect model), with error bars representing SEM. Data points show mean values

for individual mice, indicated by open circles for naïve mice and open squares for mice previously exposed to the arena. PC = pre-contact, DC = during contact.

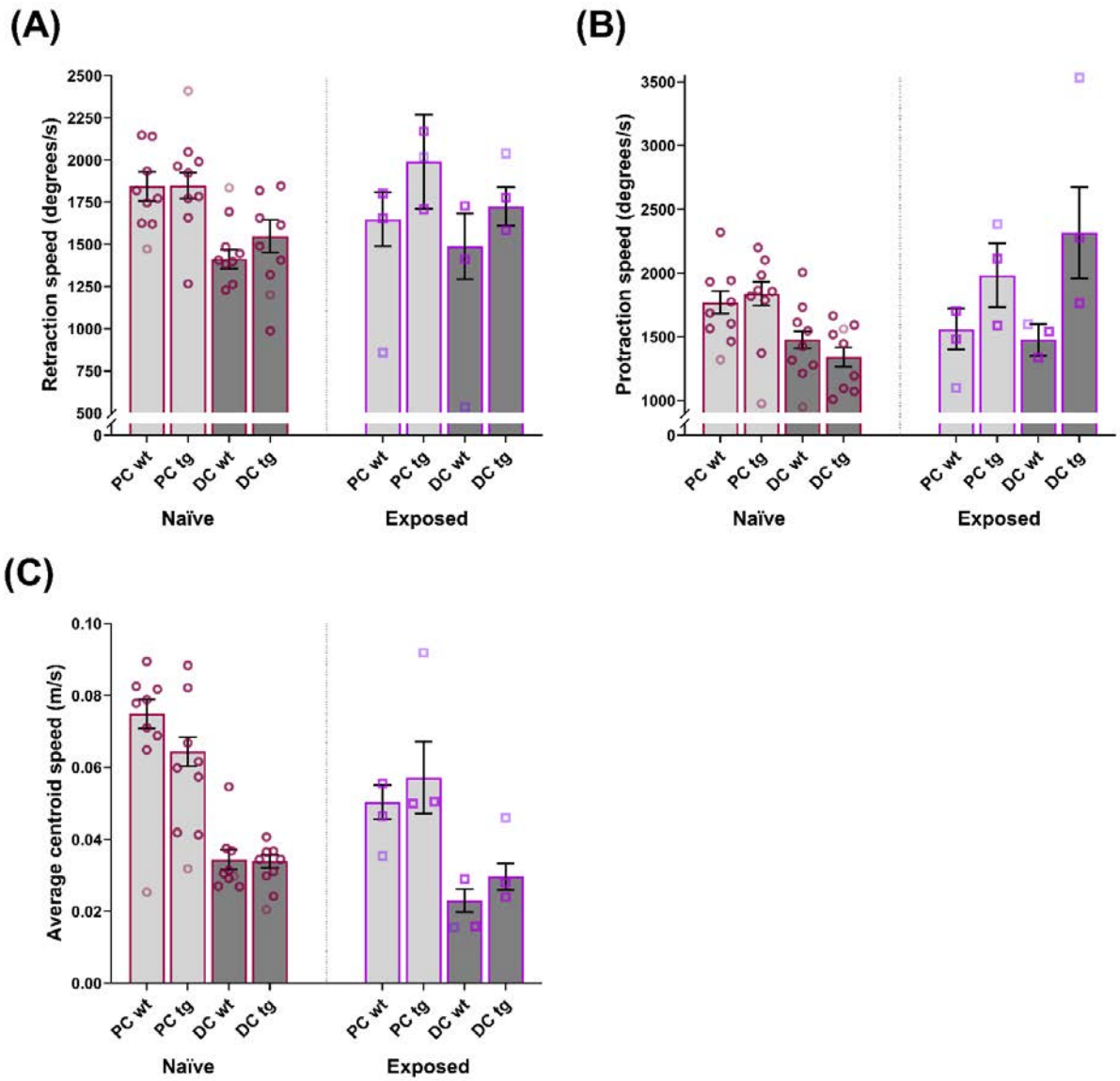


Figure 5-4 **Speed-based measures: whisker retraction speed, whisker protraction speed and average centroid (locomotion) speed.** Note the decrease in all speeds from PC to DC as a representation of the robust contact-related behaviours in naïve mice. The bars indicate the mean values from all the clips (degrees of freedom calculated from a linear mixed-effect model), with error bars representing SEM. Data points show mean values for individual mice, indicated by open circles for naïve mice and open squares for mice previously exposed to the arena. PC = pre-contact, DC = during contact.

Contact-related (PC-DC) quantitative whisker movements

The predicted robust contact-related behaviours (Table 2-1) were present in both reeler naïve and wildtype naïve mice, including an increase in amplitude and asymmetry (Figure 5-3C-D), and a decrease in locomotion speed, retraction speed, protraction speed (Figure 5-4) and spread (Figure 5-3B).

There were no genotype nor sex differences in (PC-DC) metrics, including (PC-DC) amplitude (genotype: $F(1, 11.706) = 1.4368$; sex: $F(1, 11.706) = 0.1276$; interaction $F(1, 11.706) = 0.5044$), (PC-DC) mean angular position (genotype: $F(1, 10.831) = 0.0028$; sex: $F(1, 10.831) = 1.5706$; interaction $F(1, 10.831) = 0.3062$), (PC-DC) asymmetry (genotype: $F(1, 10.54) = 1.7784$; sex: $F(1, 10.54) = 0.3957$; interaction $F(1, 10.54) = 0.5683$), (PC-DC) spread (genotype: $F(1, 11.303) = 1.1233$; sex: $F(1, 11.303) = 0.2862$; interaction $F(1, 11.303) = 0.3334$, all $p > 0.05$, Figure 5-3, Table 5-3), (PC-DC) locomotion speed (genotype: $F(1, 10.556) = 2.8249$; sex: $F(1, 10.556) = 0.3091$; interaction $F(1, 10.556) = 0.5000$), (PC-DC) retraction speed (genotype: $F(1, 10.54) = 0.3659$; sex: $F(1, 10.54) = 0.0354$; interaction: $F(1, 10.54) = 0.4862$), (PC-DC) protraction speed (genotype: $F(1, 10.55) = 1.9474$; sex: $F(1, 10.55) = 0.7139$; interaction: $F(1, 10.55) = 0.2288$, all $p > 0.05$, Figure 5-4, Table 5-3).

Habituation effect

Since there were no significant genotype effects observed in the novel object exploration task, I further investigated whisker movements in the open field over a period of five habituation sessions. Genotype ($F(1, 21.83) = 4.3695$, $p = 0.04845$), habituation ($F(2, 392.30) = 27.0910$, $p < 0.001$) and their interaction ($F(2, 392.30) = 3.7679$, $p = 0.02394$) affected whisker mean angular position. Not only was the open field different from the 1st and 5th habituation in both reeler and wildtype mice, but also the 1st and 5th habituation in reeler mice was significantly different. Specifically, open field mean angular positions were higher than those during the 5th habituation in reeler ($p < 0.0001$) and wildtype ($p = 0.0009$) mice. Similarly, open field mean angular positions were higher compared to the 1st habituation in both reeler ($p = 0.0251$) and wildtype ($p < 0.0001$) mice. Additionally, whisker

mean angular position was lower during the 5th habituation compared to the 1st habituation in reeler mice (p = 0.0132), Figure 5-5, Table 5-2).

Habituation (F (2, 389.35) = 52.7808, p < 0.0001), but not genotype (F (1, 21.93) = 2.8127, p = 0.1077) nor habituation and genotype interaction (F (2, 389.35) = 0.5817, p = 0.5594), affected locomotion speed. Specifically, locomotion speed in the 1st and 5th habituation was significantly higher than in the original open field in both reeler and wildtype mice (5th habituation compared to open field in reeler mice, p = 0.0001; 1st habituation compared to open field in reeler mice and wildtype mice, both p < 0.0001, as well as 5th habituation compared to open field in wildtype mice, p < 0.0001, Figure 5-6, Table 5-2). Similarly, habituation, but not genotype nor habituation and genotype interaction had a general effect on whisker amplitude (genotype: F (1, 21.69) = 0.2716, p = 0.60756; habituation: F (2, 394.77) = 3.4589, p = 0.03242; interaction: F (2, 394.77) = 0.8637, p = 0.42241), but post-hoc tests were not significant (Figure 5-5, Table 5-2).

There was no effect of genotype (F (1, 21.99) = 0.0310, p = 0.86182), but an effect of habituation (F (2, 386.93) = 100.0567, p < 0.001) and interaction (F (2, 386.93) = 3.4591, p = 0.03243) on whisker spread. Spread was higher in open field compared to the 1st and the 5 habituations, in both reeler and wildtype mice (all p < 0.0001). Furthermore, spread was lower during the 5th habituation compared to the 1st habituation in reeler mice (p = 0.0001, Figure 5-5, Table 5-2). There were no genotype nor habituation differences in asymmetry (genotype: F (1, 20.31) = 0.6419; habituation F (1, 358.90) = 0.5191; interaction F (1, 358.90) = 0.2078, Figure 5-5, Table 5-2), retraction speed (genotype: F (1, 21.00) = 2.0899; habituation: F (2, 392.58) = 2.8176; interaction: F (2, 392.58) = 0.7124) or protraction speed (genotype: F (1, 20.94) = 0.3760; habituation: F (2, 391.32) = 0.5017; interaction: F (2, 391.32) =0.3051), all p > 0.05 (Figure 5-6, Table 5-2).

Table 5-2 **Habituation effects.** TG = reeler, WT = wildtype mice, OF – open field. Asterisks mark significant values where $p \leq 0.05$ = *, $p \leq 0.001$ = ***.

	Genotype effect $df_1 = 1$, df_2, F, p	Habituation effect $df_1 = 2$, df_2, F, p	Genotype: Habituation interaction $df_1 = 2$, df_2, F, p	Significant post-hoc test results
Locomotion speed (m/s)	21.93 2.8127 0.1077	52.7808 389.35 < 0.0001 ***	389.35 0.5817 0.5594	TG: 1st habituation > OF TG: 5th habituation > OF WT: 1st habituation > OF WT: 5th habituation > OF
Amplitude (degrees)	21.69 0.2716 0.60756	394.77 3.4589 0.03242 *	394.77 0.8637 0.42241	All n.s.
Mean angular position (degrees)	21.83 4.3695 0.04845 *	392.30 27.0910 < 0.0001 ***	392.30 3.7679 0.02394 *	TG: 1st habituation < OF TG: 5th habituation < OF WT: 1st habituation < OF WT: 5th habituation < OF TG: 1st habituation > 5th habituation
Asymmetry (degrees)	20.31 0.6419 0.4323	358.90 0.5191 0.5955	358.90 0.2078 0.8125	All n.s.
Retraction speed (degrees/s)	21.00 2.0899 0.16303	392.58 2.8176 0.06096	392.58 0.7124 0.49110	All n.s.

Protraction speed (degrees/s)	20.94 0.3760 0.5463	391.32 0.5017 0.6059	391.32 0.3051 0.7372	All n.s.
Spread (degrees)	21.99 0.0310 0.86182	386.93 100.0567 < 0.0001 ***	386.93 3.4591 0.03243 *	TG: 1st habituation < OF TG: 5th habituation < OF WT: 1st habituation < OF WT: 5th habituation < OF TG: 1st habituation > 5th habituation

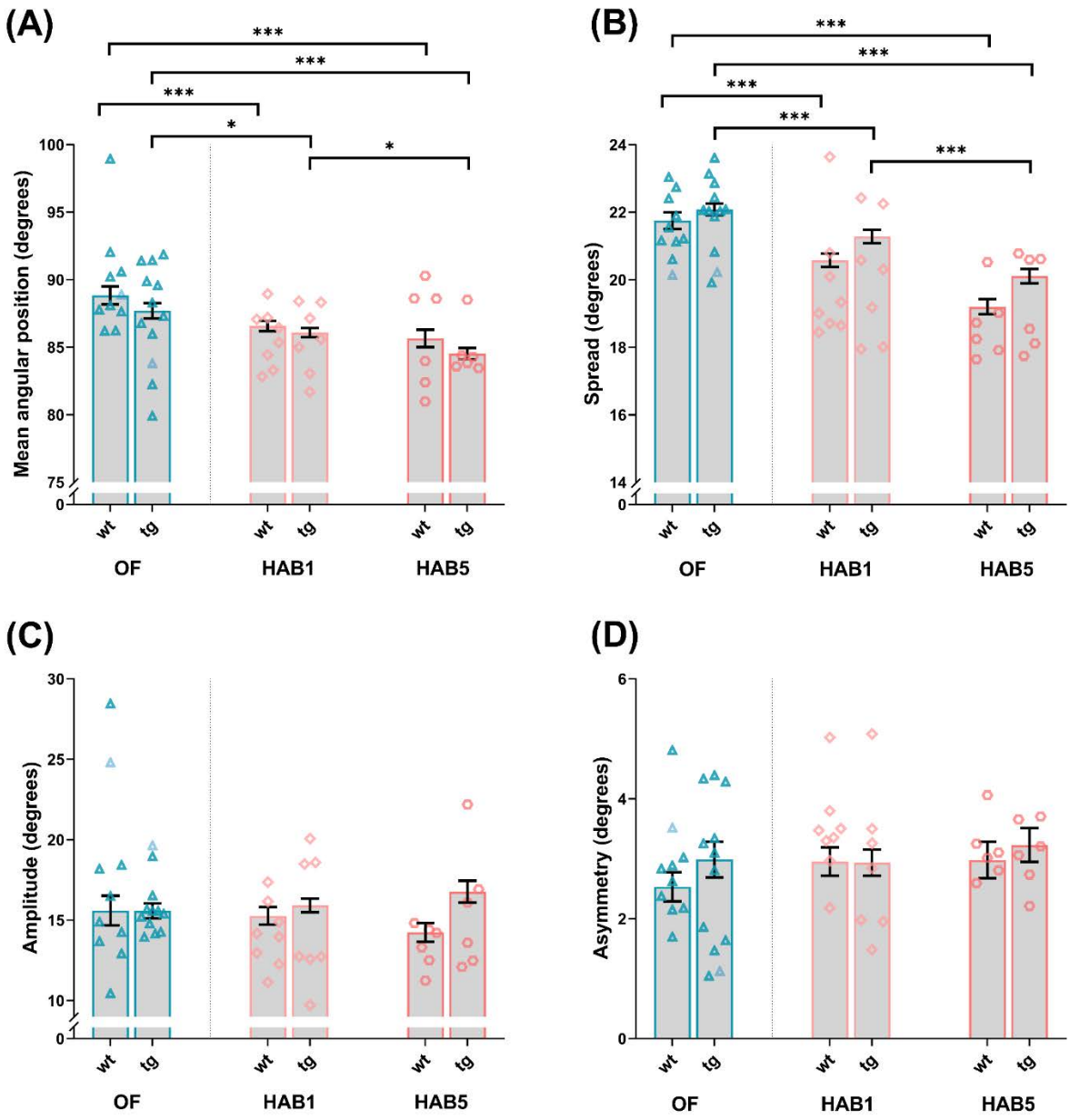


Figure 5-5 **Habituation effects on angle-based whisker measures.** Significant habituation effects were found in mean angular position (A) and spread (B). The bars indicate the mean values from all the clips (degrees of freedom calculated from a linear mixed-effect model), with error bars representing SEM. Data points show mean values for individual mice, indicated by open circles for naïve mice and open squares for mice previously exposed to the arena. OF = open filed, HAB1 = first habituation, HAB5 = 5th habituation. Asterisks mark significant values where $p \leq 0.05$ = *, $p \leq 0.001$ = ***.

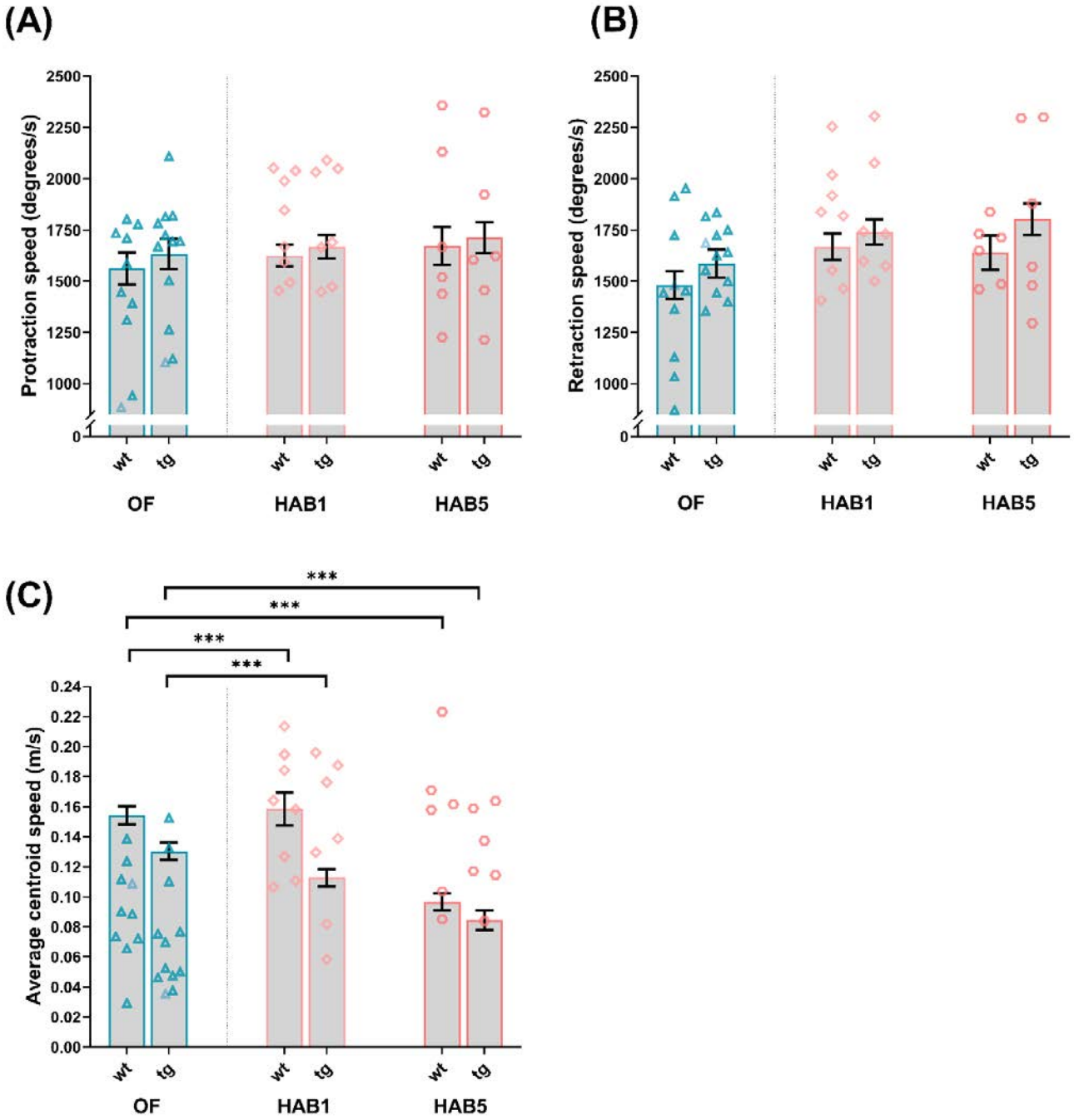


Figure 5-6 **Habituation effects on speed-based whisker and body measures.** Significant habituation effects were found in average centroid speed (C). The bars indicate the mean values from all the clips (degrees of freedom calculated from a linear mixed-effect model), with error bars representing SEM. Data points show mean values for individual mice, indicated by open circles for naïve mice and open squares for mice previously exposed to the arena. OF = open filed, HAB1 = first habituation, HAB5 = 5th habituation. Asterisks mark significant values where $p \leq 0.001$ = ***.

Nose to object distance and general behaviour

As the usual novel object exploration task resulted in additional observations of some mice not coming close enough to the object for it to be considered as contact (Figure 5-7), causing those video clips to be removed from the usual analysis, a nose-to-object distance and general arena position behaviour was measured further to investigate this. There were no significant differences between reeler and wildtype mice in the nose to object distance measure (t-test, df = 14, t = -1.0919, p = 0.2933, Figure 5-8), analysed per mouse, even though the reeler mouse nose to object distance was higher than the wildtype mice (Figure 5-8). There was also no significant difference in the percentage of time spent around the object, around the walls, or in the middle of the arena between reeler and wildtype mice in the overhead infrared video clips (Pearson‘s Chi-squared test, df = 2, X-squared = 0.84438, p = 0.6556, Figure 5-8).

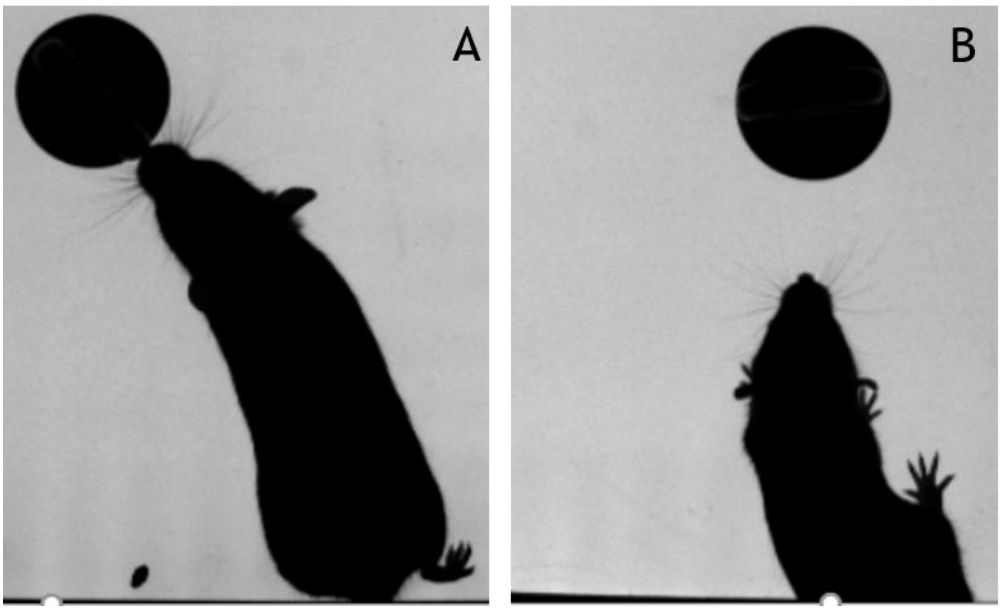


Figure 5-7 **The distance to object of wildtype (A) and *reeler* (B) mice during a novel object exploration task.** Both pictures show whiskers at their maximum protraction.

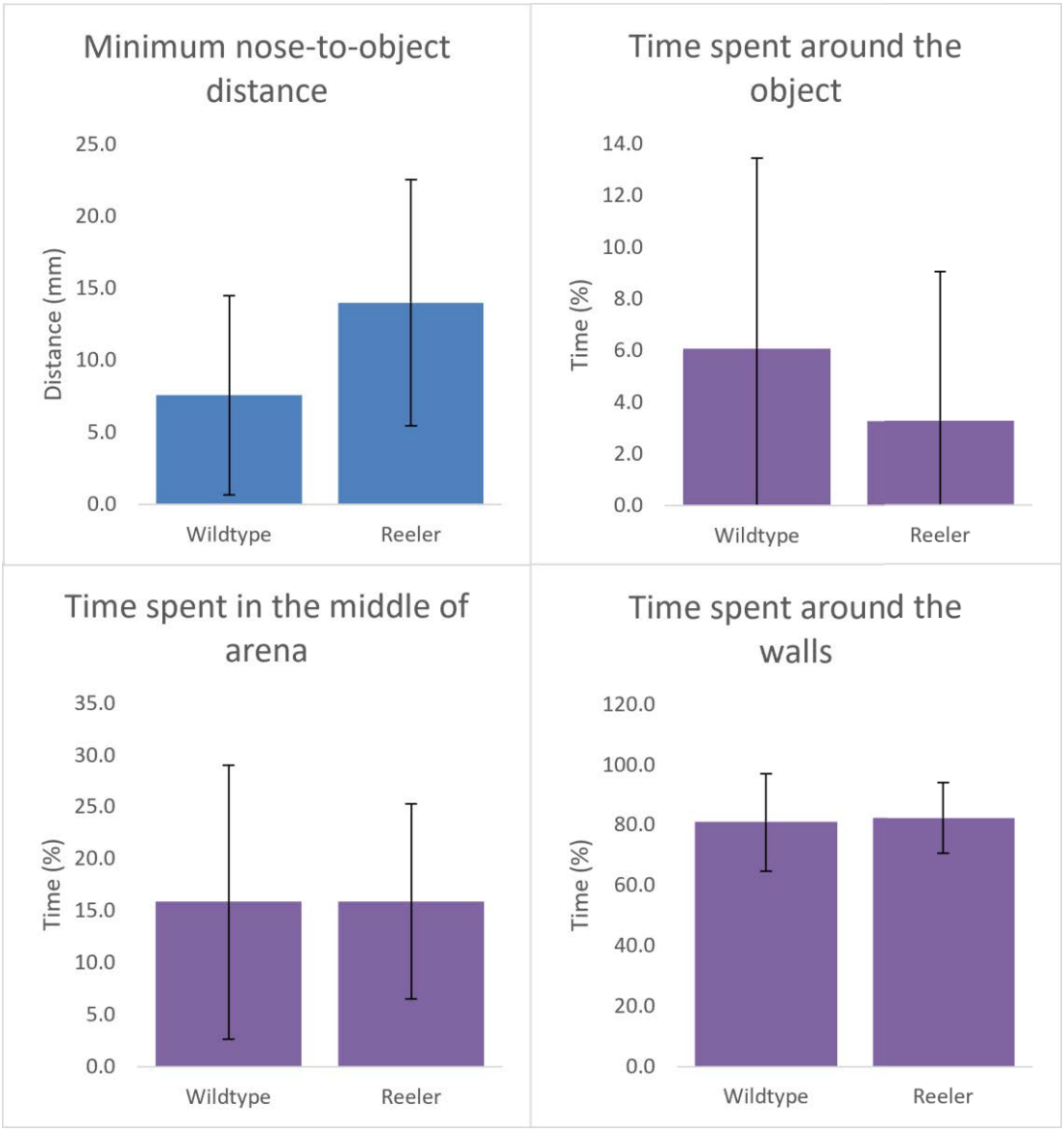


Figure 5-8 **Nose distance and general behaviour.** Nose distance measures: n = 7 wildtype and n = 9 reeler; time measures n = 12 wildtype and n = 11 reeler. Percentage of time around the object was calculated only when object was present, while time in the middle of arena and around the walls was calculated as part percentage of time in the open field and in object presence.

Neuroanatomy

There were no significant differences between reeler and wildtype mice in cell counts for sensory cortex barrel field (S1BF, t-test, df = 21.338, t = -0.27015, p = 0.7896, Figure 5-9) and corpus callosum brain areas (CC, t-test, df = 16.417, t = 1.1107, p = 0.2827, Figure 5-9). However, the cell count in motor cortex brain area

was significantly lower in reeler mice, compared to wildtype mice (M1, t-test, df = 9.9309, t = 2.8451, p = 0.01751, Figure 5-9).

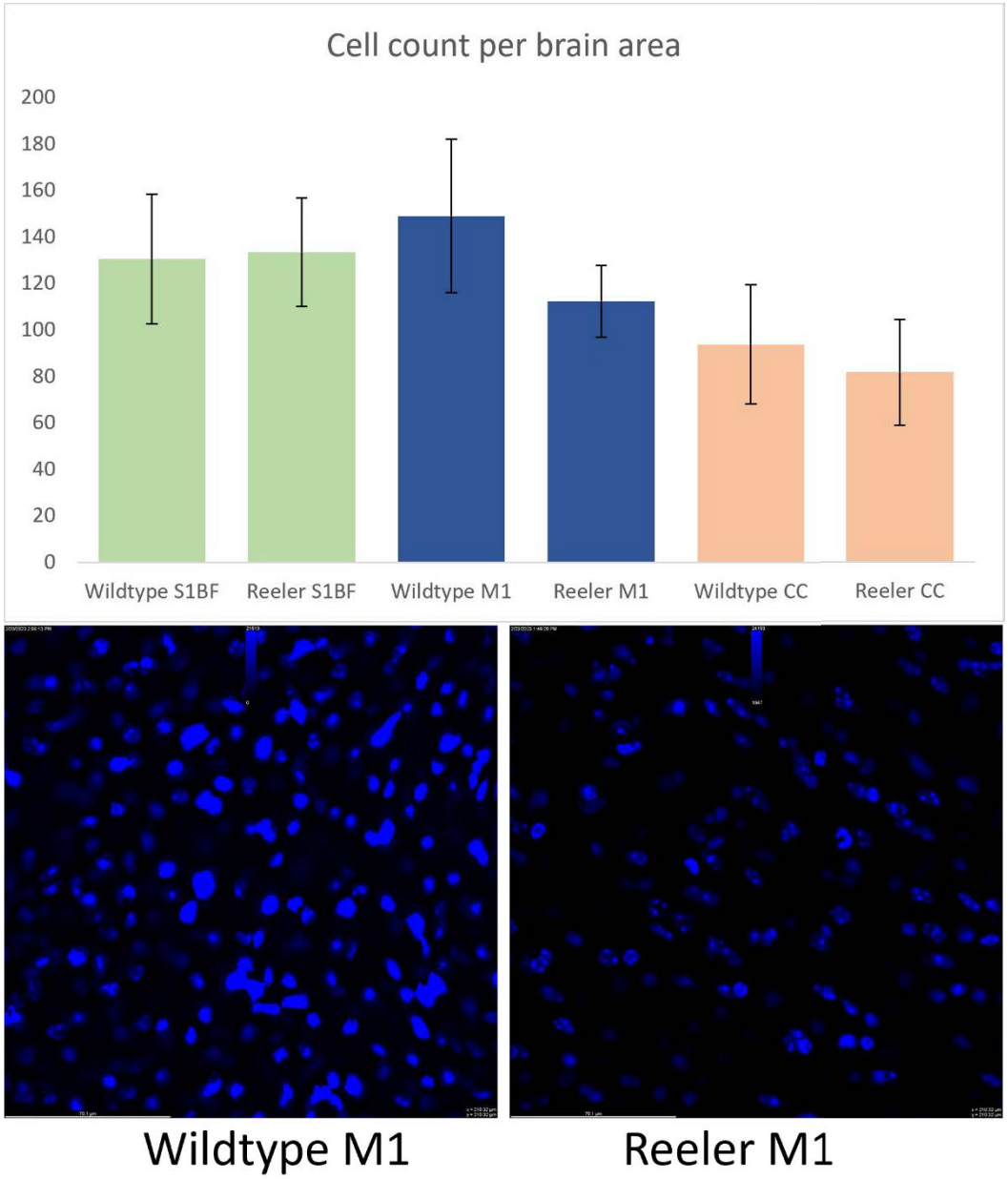


Figure 5-9 **Reeler mice have significantly lower cell count in the motor cortex (M1).** S1BF: primary somatosensory area barrel field; CC: corpus callosum. Bottom panels show an example image of M1 area. The image was taken with a x63 oil immersion objective and blue colour represents the cell nuclei. Imaging was done throughout all layers and it is not clear which cortical layer the two example pictures are from, especially in the reeler brain which did not have layers.

5.4 Discussion

Summary

Using the standard whisker protocol, only one whisker measure – pre-contact spread - was found to be affected in Genotype and Sex comparisons, without any significant differences revealed in post-hoc tests. Additional observations suggested that reeler mice often did not contact the novel object, resulting in a reduced number of contact-related clips in reeler mice, limiting the sample sizes of the during-contact (DC) whisker metrics. Habituation to the experimental arena without an object had a significant effect on locomotion speed, whisker amplitude, mean angular position and spread, although genotype differences were only observed in mean angular position, which was lower during the 5th habituation compared to the 1st habituation in reeler mice. Further experiments determined that reeler mice did not significantly differ from wildtype mice in time spent in the middle of arena, around the walls or around the object. While the nose to object measurement in reeler mice was nearly twice that of wildtype mice, this was not statistically significant either.

Novel object exploration task: previous exposure differences

There were significant differences between naïve mice and those that have been previously exposed to the testing arena. Namely, the previously exposed mice had lower PC asymmetry, higher PC spread and lower (negative) PC-DC protraction speeds, meaning that protraction speed was not reduced following a contact. The expected behaviour of a mouse upon contacting a new object is to increase whisker asymmetry and reduce whisker spread and whisking speeds (Mitchinson et al., 2007; Grant et al., 2009; Grant et al., 2018a). Here, the naïve mice followed these patterns, but the mice previously exposed to the arena did not. These whisking strategies enable mice to collect more sensory information and the focusing and positioning behaviours are also related to attention (Arkley et al., 2014; Mitchinson and Prescott, 2013). Because the mice were exposed to the arena for three consecutive days on the day before the novel object exploration

task, I assume they became habituated to the environment. However, only one out of six sessions throughout the three days involved objects, and the objects used in those sessions were different in size, texture and shape from the glass bottle stopper used in the novel object exploration study. It is not clear why the behaviour of mice would differ significantly in this case and why these differences would only manifest in the novel object exploration task and not the open field task. Perhaps habituation to arena has a significant impact on many behaviours and could be a useful tool when looking for differences in mouse behaviour. This will be further reviewed in the Discussion chapter of this thesis (Chapter 6).

Pre-contact whisker movements

I previously assessed the whisking and exploratory behaviours of reeler mice in the pre-contact portion of the novel object exploration task (Simanaviciute et al., 2020) and found that the PC mean angular position was significantly higher in heterozygous reeler than in wildtype mice. PC mean angular position was not significantly affected in the reeler mice in this current study, however, the age and genetics of the mice here were different from those in the previous one, where I tested heterozygous reeler mice of 7-9 months of age. Rather, here, I found that PC whisker spread may be significantly affected in the reeler mice, although there were no significant post-hoc comparisons, and whisker spread was not measured in the previous paper (Simanaviciute et al., 2020).

Indeed, it is surprising that such disrupted brain anatomy in the reeler mice (Hafner et al., 2021) can give rise to only subtle behavioural differences. When I examined the brain anatomy of the whisker-related areas M1, S1BF and CC, significant reductions were found in cell counts in the motor cortex, as supported by Hafner et al. (2021) study which involved larger sample sizes and expert neuroanatomical methods. While ablation of the motor cortex has been found to affect whisker asymmetry, amplitude and speeds (Gao et al. 2009), I found genotype differences in PC spread and in mean angular position in the open field habituation studies. Perhaps much of the motor cortex is left intact in reeler mice, despite the reduction in cell counts, and compensatory brain mechanisms could

also probably protect the whiskers from being largely impacted, since they are such an important sense in mice.

Contact-related whisker movements

There were no significant effects of genotype (reeler vs. wildtype) in the PC-DC measures. It is exceedingly rare to not find an effect in these metrics and thus far has only occurred in scPCP rats (Landreth et al., 2021). There are, however, multiple mouse models that do show differences between the model and wildtype mice in this exact set up and task, such as 5xFAD model of Alzheimer's disease (Grant et al., 2018b) and 3xTg-AD model of Alzheimer's disease (Simanaviciute et al., 2022).

Since there were lower sample numbers in the object exploration task for reeler mice (27 clips), compared to the wildtype mice (46 clips), it introduced a question of whether reeler mice were avoiding or not getting close enough to the object which would result in a reduced sample size after clip selection. To address this, a new behavioural set-up was developed by the addition of an overhead camera. Using this, I found that reeler mice spent the same length of time around the object as control mice. However, the distance to the object measure from the high-speed video footage showed that reeler mice tended not to get as close to the object as wildtype mice, although this was not statistically significant. Small changes in distance to the object are likely to increase the chances of the animals not touching the object with their whiskers (Figure 5-7) and that could still affect which clips were included in the usual analysis, despite that measure not being statistically different. This is an important consideration for standardising the protocol and will be further reviewed in this chapter in 5.5 Method development section.

Open field

Due to the visible ataxia and motor impairments that reeler mice exhibit, it was surprising to see no differences between reeler and wildtype mice neither in locomotion speed nor in any whisking behaviours in the open field. There is a

possibility of a bias when selecting the video clips for those mice that have severe motor disturbances, especially in reeler mice, known for reeling behaviour. One of the selection criteria for open field clips is that mice must keep their head in level with the floor and the clips captured were just over one second long. This presents the possibility that only lesser-impacted episodes made it to the open field analysis, and hence the differences captured in the analysis were not significant. While reeler mice did not show impacted whisker and locomotor behaviour in the open field arena, many mouse models have shown significant differences between the model and wildtype mice in the same open field set-up, including the SOD1 model of Amyotrophic Lateral Sclerosis, R6/2 model of Huntington's disease (with sex differences in protraction and retraction speeds) and MCAO model of ischaemic stroke (Simanaviciute at al., 2020).

Habituation effect

To build on my observation that whisker movements were affected by previous exposure to the arena, mice were filmed with a high-speed camera during a further habituation period. Habituation was found to affect mean angular position and spread in reeler mice, where during the 1st habituation both mean angular position and spread were higher than in the 5th habituation. As for most (18) mice the open field session happened before the additional habituation sessions, it is also worth looking at the changes occurring from open field to the 1st habituation to then the 5th habituation. With that in mind, additional habituation affected both wildtype and reeler mice with mean angular position, spread and locomotion speed all generally decreasing over the habituation period. This means that, following habituation, animals were moving slower, they were holding their whiskers further back and more spread out, which might suggest that the animals were not as motivated to explore the arena. In particular, whisker angular position in reeler mice decreased consistently between the open field and the 1st and 5th habituation, suggesting that they were less focused on the area ahead of themselves as they got more familiar with the environment (Arkley et al., 2014). The effect of habituation on whisker movements has not really been investigated before, although Arkley et al.

(2014) found that, in sighted rats, increasing familiarity of the experimental set up while training to perform a task resulted in increased locomotion speed, more forward whiskers and increased whisker amplitude. This is the opposite of my findings of decreased locomotion speed and angular position and likely points to the behavioural differences that occur between tasks. For example, Arkley et al. (2014) trained rodents using food rewards to complete a specific task in a changing environment, while results discussed in this chapter came from tasks without animal training and no environmental changes. Nevertheless, running multiple sessions and observing whisker movement changes during a habituation period might be a useful task to add to our usual procedures. This will be an important consideration for the protocol standardisation further detailed in the Discussion Chapter 6 of this thesis.

5.5 Method development

Since the usual object exploration task only revealed genotype effects on the PC measure of spread, and no genotype effects on any contact-related (PC-DC) whisker movements, I conducted a supplementary study looking at habituation to the arena. This identified an additional genotype effect in mean angular position and spread, and habituation effects in many of the whisker variables. This is the first study to look at habituation in whisker movements and is an exciting avenue for future research. I suggest that the standard whisker measurement protocol should be adapted to incorporate other tasks to suit the specific rodent model, especially if there are no significant findings in the usual task, and if observations during the usual task can provide evidence for an additional measure. For example, here I saw that animals that had been exposed to the arena before behaved differently, which was my motivation for including the habituation study.

I suggested that the lack of findings in the PC-DC measures may be due to fewer clips being collected of the reeler mice interacting with the object. Indeed, I observed the reeler mice often not contacting the object with their whiskers, despite being close to it. To investigate this further, I installed an overhead infrared camera to monitor the position of the mice throughout the whole trial. This gave a

lot of insight into their behaviour, compared to my usual data collection, which only collects snippets of videos of ~1 second, rather than a full ~10-minute video for each trial. The reeler mice all spent the same amount of time around the object, compared to the wildtype mice. And despite often being further from the object, this was not a statistically significant finding. The addition of the overhead camera provides a useful extra monitoring system alongside the high-speed video footage. It can also offer flexibility, allowing researchers to collect additional data after the trials have been conducted, such as the position of the mouse in the arena, their proximity to the object, or number of interactions with the object.

5.6 Conclusion

This work has contributed to the third PhD objective – integrating whisker movement protocol with other behavioural tasks and placing them within the context of general exploratory behaviour. While I still think that whisker movements are a powerful behavioural measurement tool, my observations in reeler mice suggest that making measurements in a standardized manner might not suit all mouse models if their impairment does not allow for a close contact with the object. Incorporating whisker measurements within a further battery of behavioural tests allows detection of additional exploratory-related parameters. Therefore, further work should consider incorporating whisker movements into more common behavioural tests to complement standard tasks that assess object exploration and habituation in particular.

5.7 Supplementary material

Table 5-3 **Novel object exploration results**. Main results are reported for all whisker measures. Additional post-hoc analyses and means are reported for PC spread.

> #PC average centroid speed						
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Genotype	0.00099528	0.00099528	1	9.0048	1.8860	0.2029
Line	0.00033563	0.00016782	2	9.1210	0.3179	0.7354
Genotype:Line	0.00004132	0.00002066	2	9.1210	0.0391	0.9618
> #Log PC amplitude						

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Genotype	0.006267	0.0062668	1	8.9047	0.3336	0.5778
Line	0.005770	0.0028852	2	9.0212	0.1536	0.8598
Genotype:Line	0.032104	0.0160518	2	9.0212	0.8544	0.4573

> #Tuk PC mean angular position

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Genotype	6.0935e+12	6.0935e+12	1	7.4986	0.4386	0.5276
Line	7.4973e+12	3.7486e+12	2	7.5948	0.2697	0.7706
Genotype:Line	2.4464e+13	1.2232e+13	2	7.5948	0.8800	0.4532

> #Sqrt PC asymmetry

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Genotype	0.18330	0.183305	1	6.9018	0.4069	0.5441
Line	0.52823	0.264116	2	6.9716	0.5858	0.5819
Genotype:Line	0.04005	0.020027	2	6.9716	0.0444	0.9568

> #PC retraction speed

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Genotype	6775	6775	1	7.6787	0.0276	0.8724
Line	3943	1971	2	7.7803	0.0080	0.9920
Genotype:Line	92961	46481	2	7.7803	0.1892	0.8313

> #PC protraction speed

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Genotype	49237	49237	1	6.9018	0.1666	0.6955
Line	115895	57947	2	6.9716	0.1960	0.8264
Genotype:Line	1115376	557688	2	6.9716	1.8860	0.2215

> #Tuk PC spread

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Genotype	993515555	993515555	1	9.4032	2.9525	0.1184
Line	1476061792	738030896	2	9.5170	2.1930	0.1647
Genotype:Line	45550927	22775463	2	9.5170	0.0677	0.9350

#PCDC average centroid speed

Type III Analysis of Variance Table with Kenward-Roger's method

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Genotype	0.00123468	0.00123468	1	6.9215	1.5307	0.2563
Line	0.00171432	0.00085716	2	6.9924	1.0618	0.3956
Genotype:Line	0.00005487	0.00002743	2	6.9924	0.0340	0.9667

> #PCDC amplitude

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Genotype	47.770	47.770	1	9.3674	1.3641	0.2717
Line	12.175	6.088	2	9.4815	0.1738	0.8431

Genotype:Line 25.746 12.873 2 9.4815 0.3675 0.7019
> #PCDC mean angular position
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 16.940 16.940 1 6.9018 0.7684 0.4102
Line 139.810 69.905 2 6.9716 3.1685 0.1050
Genotype:Line 52.364 26.182 2 6.9716 1.1867 0.3601
> #Tuk PCDC asymmetry
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 6124.9 6124.9 1 7.3311 1.9390 0.2046
Line 3816.2 1908.1 2 7.4211 0.6037 0.5716
Genotype:Line 1456.1 728.1 2 7.4211 0.2303 0.7997
> #PCDC retraction speed
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 198145 198145 1 7.1736 0.4475 0.5245
Line 47519 23760 2 7.2570 0.0536 0.9482
Genotype:Line 2 0.6538
> #PCDC protraction speed
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 504713 504713 1 7.6301 1.2664 0.2946
Line 336487 168243 2 7.7304 0.4220 0.6700
Genotype:Line 1823 912 2 7.7304 0.0023 0.9977
> #Tuk PCDC spread
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 15.2342 15.2342 1 8.2745 3.5784 0.09396 .
Line 1.4924 0.7462 2 8.3878 0.1752 0.84226
Genotype:Line 17.8507 8.9253 2 8.3878 2.0959 0.18272
> #PC average centroid speed
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 0.00143817 0.00143817 1 11.427 2.7201 0.1263
Sex 0.00004259 0.00004259 1 11.427 0.0806 0.7816
Genotype:Sex 0.00036745 0.00036745 1 11.427 0.6950 0.4216
> #Log PC amplitude
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 0.0102537 0.0102537 1 11.585 0.5489 0.4735
Sex 0.0033942 0.0033942 1 11.585 0.1817 0.6777
Genotype:Sex 0.0072492 0.0072492 1 11.585 0.3880 0.5454
> #Tuk PC mean angular position
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)

Genotype 2.5070e+13 2.5070e+13 1 10.54 1.9549 0.19078
Sex 4.2354e+13 4.2354e+13 1 10.54 3.3027 0.09767 .
Genotype:Sex 5.2278e+13 5.2278e+13 1 10.54 4.0765 0.06965 .
> #Sqrt PC asymmetry
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 0.73616 0.73616 1 10.54 1.7344 0.2158
Sex 1.00555 1.00555 1 10.54 2.3691 0.1532
Genotype:Sex 0.61421 0.61421 1 10.54 1.4471 0.2553
> #PC retraction speed
Type III Analysis of Variance Table with Kenward-Roger's method
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 48282 48282 1 10.54 0.1981 0.6653
Sex 134057 134057 1 10.54 0.5499 0.4746
Genotype:Sex 331557 331557 1 10.54 1.3600 0.2692
> #PC protraction speed
Type III Analysis of Variance Table with Kenward-Roger's method
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 85303 85303 1 10.54 0.2877 0.6029
Sex 266049 266049 1 10.54 0.8972 0.3648
Genotype:Sex 250334 250334 1 10.54 0.8442 0.3788
> #Tuk PC spread
Type III Analysis of Variance Table with Kenward-Roger's method
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 1790840304 1790840304 1 12.057 5.4403 0.03781 *
Sex 65883384 65883384 1 12.057 0.2001 0.66254
Genotype:Sex 828729162 828729162 1 0.13845

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> lsmeans(m.mixed.TukPC.spread,pairwise~Genotype*Sex)
$lsmeans
Genotype Sex lsmean SEM df lower.CL upper.CL
TG F 66893 10330 11.0 44162 89625
WT F 35900 7392 11.5 19711 52089
TG M 50807 6118 14.1 37694 63921
WT M 44910 7168 13.9 29524 60295
Degrees-of-freedom method: kenward-roger
Confidence level used: 0.95
$contrasts
contrast estimate SEM df t.ratio p.value
TG F - WT F 30993 12703 11.2 2.440 0.1255

TG F - TG M 16086 12006 11.7 1.340 0.5575
TG F - WT M 21983 12574 11.8 1.748 0.3433
WT F - TG M -14908 9595 12.4 -1.554 0.4375
WT F - WT M -9010 10297 12.6 -0.875 0.8176
TG M - WT M 5898 9424 14.0 0.626 0.9220
Degrees-of-freedom method: kenward-roger
P value adjustment: tukey method for comparing a family of 4 estimates
> #PCDC average centroid speed
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 0.00226090 0.00226090 1 10.556 2.8249 0.1221
Sex 0.00024735 0.00024735 1 10.556 0.3091 0.5898
Genotype:Sex 0.00040019 0.00040019 1 10.556 0.5000 0.4948
> #PCDC amplitude
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 50.700 50.700 1 11.706 1.4368 0.2544
Sex 4.502 4.502 1 11.706 0.1276 0.7273
Genotype:Sex 17.798 17.798 1 11.706 0.5044 0.4915
> #PCDC mean angular position
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 0.062 0.062 1 10.831 0.0028 0.9589
Sex 35.296 35.296 1 10.831 1.5706 0.2365
Genotype:Sex 6.882 6.882 1 10.831 0.3062 0.5912
> #Tuk PCDC asymmetry
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 5621.1 5621.1 1 10.54 1.7784 0.2104
Sex 1250.7 1250.7 1 10.54 0.3957 0.5427
Genotype:Sex 1796.1 1796.1 1 10.54 0.5683 0.4675
> #PCDC retraction speed
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 160605 160605 1 10.54 0.3659 0.5581
Sex 15527 15527 1 10.54 0.0354 0.8544
Genotype:Sex 213411 213411 1 10.54 0.4862 0.5007
> #PCDC protraction speed
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 780178 780178 1 10.55 1.9474 0.1915
Sex 286007 286007 1 10.55 0.7139 0.4169
Genotype:Sex 91667 91667 1 10.55 0.2288 0.6422
> #Tuk PCDC spread

Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 4.8539 4.8539 1 11.303 1.1233 0.3113
Sex 1.2367 1.2367 1 11.303 0.2862 0.6030
Genotype:Sex 1.4407 1.4407 1 11.303 0.3334 0.5750
> #PC average centroid speed
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 0.00072630 0.00072630 1 8.5545 1.3753 0.2725
Months 0.00017666 0.00005889 3 8.4603 0.1114 0.9511
Genotype:Months 0.00058814 0.00029407 2 8.6876 0.5564 0.5924
> #Log PC amplitude
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 0.0199378 0.0199378 1 8.6364 1.0640 0.3303
Months 0.0213584 0.0071195 3 8.5492 0.3797 0.7703
Genotype:Months 0.0087446 0.0043723 2 8.7734 0.2332 0.7968
> #Tuk PC mean angular position
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 1.3625e+13 1.3625e+13 1 7.9007 0.9842 0.3506
Months 8.3455e+12 2.7818e+12 3 7.7274 0.2006 0.8930
Genotype:Months 2.7867e+13 1.3933e+13 2 7.9593 1.0048 0.4082
> #Sqrt PC asymmetry
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 0.43327 0.43327 1 7.5602 1.0024 0.3477
Months 0.93150 0.31050 3 7.3047 0.7164 0.5717
Genotype:Months 1.97341 0.98670 2 7.5153 2.2763 0.1687
> #PC retraction speed
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 14434 14434 1 7.5602 0.0590 0.8146
Months 120413 40138 3 7.3047 0.1635 0.9177
Genotype:Months 978334 489167 2 7.5153 1.9923 0.2022
> #PC protraction speed
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 880 880 1 7.5815 0.0029 0.9583
Months 751512 250504 3 7.3332 0.8289 0.5171
Genotype:Months 350311 175156 2 7.5461 0.5796 0.5833
> #Tuk PC spread
Sum Sq Mean Sq NumDF DenDF F value Pr(>F)
Genotype 369625722 369625722 1 9.2176 1.1135 0.3182
Months 504529284 168176428 3 9.1696 0.5064 0.6873
Genotype:Months 58800081 29400040 2 9.3618 0.0885 0.9160

```
> #PCDC average centroid speed
                  Sum Sq    Mean Sq NumDF  DenDF F value Pr(>F)
Genotype        0.00069063 0.00069063     1 7.5866  0.8582 0.3828
Months          0.00156439 0.00052146     3 7.3399  0.6463 0.6086
Genotype:Months 0.00136388 0.00068194     2 7.5533  0.8451 0.4664

> #PCDC mean angular position
                Sum Sq Mean Sq NumDF  DenDF F value Pr(>F)
Genotype         13.569  13.569     1 7.5602  0.6046 0.4605
Months          102.628  34.209     3 7.3047  1.5201 0.2882
Genotype:Months 119.925  59.963     2 7.5153  2.6642 0.1335

> #Tuk PCDC asymmetry
                Sum Sq Mean Sq NumDF  DenDF F value Pr(>F)
Genotype         4581.9  4581.9     1 7.6788  1.4508 0.2642
Months           7671.1  2557.0     3 7.4587  0.8078 0.5263
Genotype:Months  521.1   260.6      2 7.6799  0.0823 0.9218

> #PCDC retraction speed
                Sum Sq Mean Sq NumDF  DenDF F value Pr(>F)
Genotype         220260  220260     1 7.5785  0.4906 0.5046
Months           414277  138092     3 7.3292  0.3068 0.8200
Genotype:Months  90798   45399      2 7.5418  0.1008 0.9053

> #PCDC protraction speed
                Sum Sq Mean Sq NumDF  DenDF F value Pr(>F)
Genotype         185111  185111     1 7.7045  0.4674 0.5142
Months           823367  274456     3 7.4909  0.6915 0.5840
Genotype:Months 123841   61920      2 7.7138  0.1560 0.8582

> #Tuk PCDC spread
                Sum Sq Mean Sq NumDF  DenDF F value Pr(>F)
Genotype         7.0148  7.0148     1 8.2517  1.6510 0.2337
Months           2.7732  0.9244     3 8.1274  0.2173 0.8817
Genotype:Months 16.5624  8.2812     2 8.3617  1.9470 0.2022
```

CHAPTER 6 DISCUSSION

6.1 Findings

Research findings in disease models

In Chapter 3, I described the earliest motor phenotype I ever observed by tracking whiskers in 3xTg AD mice at only 3 months of age, manifesting as a reduction in whisker angles. Additionally, pre-contact whisking amplitude at 17 months differed between the 3xTg-AD and wildtype mice. During object contact 3xTg-AD mice did not reduce whisker spread as frequently as the wildtype mice at 12.5 and 17 months, which may suggest sensory or attentional deficits. Previous studies have not shown any differences at the latest stages of disease in this mouse model (not significant at 15 months in Stevens and Brown (2015) and Fertan et al. (2019), Table 3-5). Whisker movement abnormalities in Alzheimer's disease could be related to amyloid plaques, as those were detected in the barrel cortex of some AD mouse models, thus, I recommend using this set up in more AD models to understand if amyloid levels are affecting whisker movements specifically in AD models. It does seem like whisker movement measures are particularly well-suited for showing behavioural deficits in AD mice and has also been demonstrated in 5xFAD mice (Grant et al. 2020).

In Chapter 4, I showed whisker movement deficits for the first time in a rat model, and treatment effects for the first time in a sequential object task in Poly I:C model. Specifically, Poly I:C treatment in rat dams affected their female adult offspring, who did not increase whisker mean angular position during object exploration, indicating an attentional deficit. Animal sex and object type were also found to significantly affect whisker movements in the maternal immune activation model. Whisker tracking during sequential object exploration was demonstrated to be powerful enough to detect both treatment and sex effects into adulthood in this model of neurodevelopmental disorders.

Finally, in Chapter 5, when studying the reeler mouse model, I found that habituation affected locomotion speed, whisker amplitude, mean angular position and spread in both wildtype and reeler mice. This is the first time that habituation has been found to affect whisker movements and is a useful task to consider for future studies, since it allowed to detect behavioural deficits in this subtle behavioural model when the standard protocol did not work.

Overall, I have found that whisker movements can be measured during naturalistic tasks, such as in the open field and during novel object exploration. It can offer a refinement to animal testing by providing quantitative data, without the need for training, reward, or habituation However, while I believe that whisker movements remain a powerful behavioural measurement tool, the usual protocol might not suit all mouse models, especially if their impairment does not allow for a close contact with the object or does not manifest in a deficit in exploratory behaviour.

Addressing thesis aims and objectives

The aim of my PhD work is to make recommendations for the application of whisker movement measures for the study of rodent models of neurodegeneration, especially in i) standardisation, ii) automation, and iii) placing whiskers in the context of other tests. I consider each of these aspects below.

From my literature review in Chapter 2, I also identified the need to: i) establish if the protocol will work in rats; ii) adapt statistical approach to reflect the repeated contacts with the same object; and iii) investigate sex differences. These have all been achieved in my thesis and will remain part of the experimental protocol.

i) Standardisation

I recommend using the protocol developed through my thesis (in more detail below). This protocol is suitable to use in both mice and rats, and it is probably even applicable for use with other small, common laboratory animals, such as hamsters and grey short-tailed opossums. I recommend that males and females should be included where possible, and based on my observations, around ten

animals in each group should work well statistically, but this can also be confirmed using sample size calculations. The statistical techniques I adopted (LMEM with Kenward-Roger's method for degrees of freedom) makes the most of smaller sample sizes, by providing a balance in sample numbers between the number of animals and the number of clips present in the analysis. I strongly recommend using LMEM with Kenward-Roger's method for degrees of freedom as the statistical approach with this protocol. This is especially relevant now after Chapter 4 findings where I showed that habituation influences whisker movements. Considering that every subsequent touch to the same object is slightly different with the increase of information collected, automated degrees of freedom should be used. Treating every video clip as a degree of freedom is strongly discouraged and will be statistically incorrect. Averaging all videos per animal might be another approach, however, this might need more animals to reach the intended power of analysis. I have also improved the automation of the protocol by removing the qualitative scoring steps and including an additional quantitative measure of whisker spread.

If previous studies in the model are available and have revealed deficits in a particular group of animals, such as how it did in younger 3xTg-AD female mice, this is a good indication to record more videos from that group. As it happened in Chapter 3 and Chapter 5, some animals will be more prone to behaving in a way where clips do not pass the criteria for tracking. The pilot study and previous work will inform of this possibility and, therefore, it might be worth spending more time collecting more data from those groups of animals.

My protocol is detailed in a step-by-step fashion below (Figure 6-1, and text below). Overall, I recommend adopting this protocol and moving away from more complicated tasks requiring habituation and training, which can cause experimenter-related variation. Instead, I suggest performing quick studies which reduce day-to-day environmental variation and include more quantitative measures. However, should the quick tasks not reveal any differences in treatment, then habituation and sequential object tasks could be investigated, as I did in Chapters 4 and 5. See the recommended protocol below in Figure 6-1.

I would recommend keeping the whisker filming set up in the same place throughout the length of the full experiment and to do testing as quickly as possible. To reduce individual differences in behavioural cross-sectional studies, a longitudinal study in the same animals should be conducted if disease progression is of interest and the model allows for good sample sizes at later disease stages. However, large gaps of time are needed between testing in such cases because it might be difficult to separate treatment from habituation effects if testing repeatedly without those considerable breaks. Males and females should be included analysed separately, using sex-based reporting (Yoon et al., 2014, Miller et al., 2017). So far, I have seen baseline differences in PC mean angular position between male and female control rats.

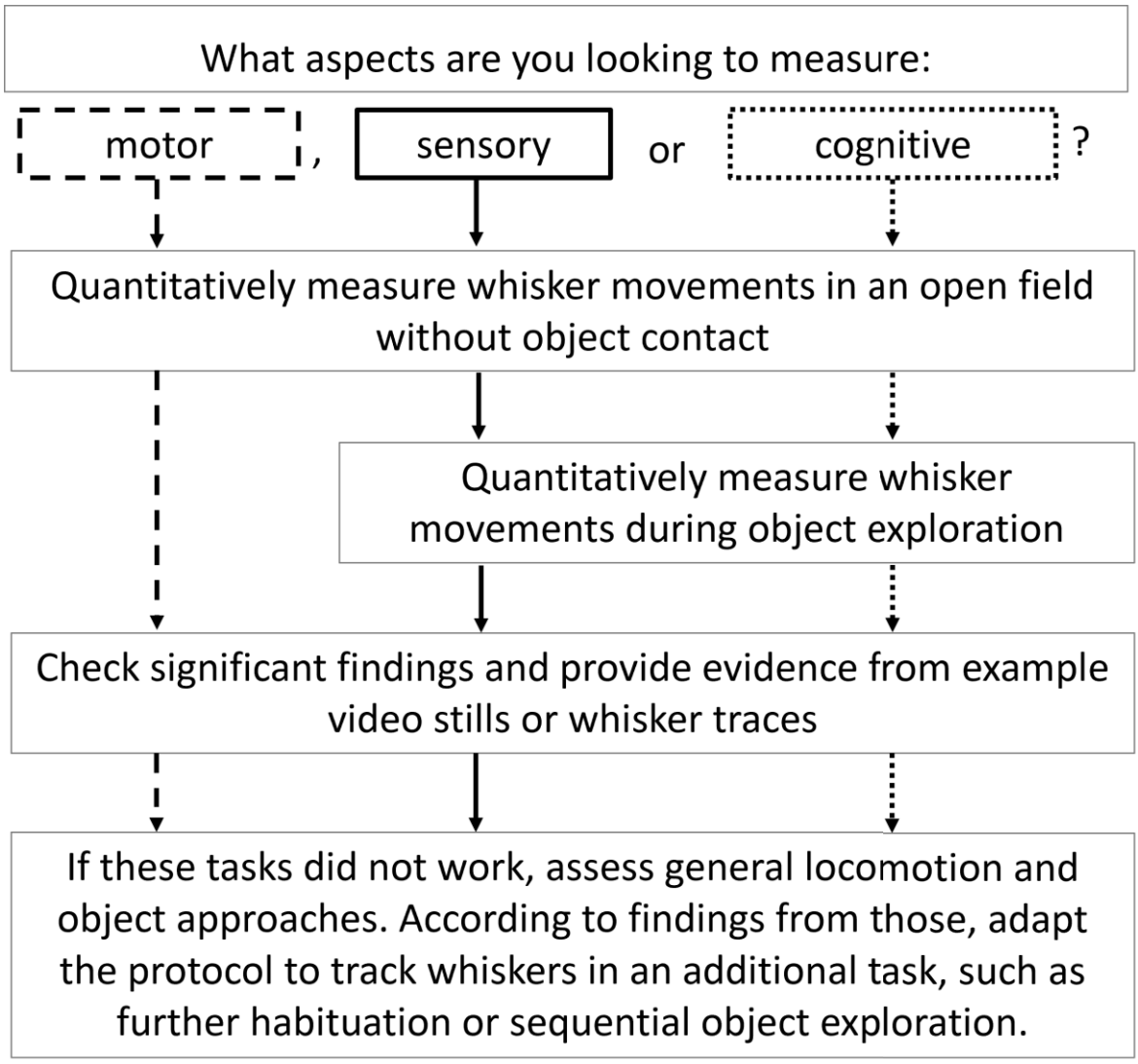


Figure 6-1 **The final recommended workflow for whisker movement studies**, adjusted for conclusions made in the thesis. If it is not clear whether you expect motor, sensory or cognitive effects from your disease model, apply all steps.

The protocol

This protocol focuses on mice and rats in the laboratory; however, please see a recent publication by Grant et al. (2023) for how the protocol can be adapted to work with any small mammal species. Use PREPARE guidelines (Smith et al., 2018) to plan the experiment well before the start of the data collection and the 3Hs project guidelines on housing, handling and habituation ("The 3Hs Initiative," n.d.).

Data collection

1. Animals should be filmed during their active phase. Nocturnal animals, like mice and rats, are ideally filmed in a dark room during their active phase. Even under a dim red light, animals rely on visual cues in object exploration (Hu et al., 2018), thus, if purely tactile performance is of interest, data collection should be conducted in the dark for mice and rats.
2. Animals must not have previous exposure to the same experimental arena since they are affected by habituation. They may be tested in other set ups before.
3. Light box: ideally infrared or near-infrared; if not, white light with an IR filter. The size of the light box should be adjusted according to the size of the animals; for example, it should be larger for rats.
4. Arena size should be chosen according to the size of the species being studied. A good reference for both rats and mice is 30 × 50 × 15 cm
5. Recording speed: ideally 500 fps, if not, around 300 fps
6. Resolution: minimum 640 x 480 pixels
7. Number of animals: minimum 10 per group, including males and females
8. Check whiskers for barbering; if barbered, house alone for 2 weeks prior to testing or exclude those animals if single housing is not preferred
9. Film a ruler for calibration every time there are changes to the camera set up
10. Include object exploration if studying sensory or cognitive deficits. Consider object texture when choosing and object, as this may affect whisker movements

11. Ideally, combine with additional infrared camera recording whole trials that are then analysed by other software such as BORIS (Friard and Gamba, 2016) used in this thesis
12. Conduct a pilot study of standard open field / object method; add adjustments if needed for the particular rodent model; e.g., a habituation study, sequential object task

Video processing

1. Select clips according to the criteria: i) the head clearly in frame; ii) both sides of the face visible; iii) the head level with the floor (no extreme pitch or yaw); iv) animal must be travelling toward the object in the pre-contact (PC) section of the clip; v) the whiskers only contact the object and not the vertical arena walls in the during-contact (DC) section of the clip.
2. Track whole clips in ARTv2, saving the .awrt files
3. Review tracking and save only the PC portion, note the frame
4. Review tracking and save only the DC portion, start on the noted frame +1
5. Convert pixels to millimetres using calibration from filming the ruler and export results

Statistical analysis and reporting

1. Calculate sample sizes after tracking (if not using automated approach such as Kenward-Roger's method for degrees of freedom)
2. Check if any animals have only 1-2 clips, remove, if needed, e.g. if choosing a different statistical method which cannot cope with such numbers as well as LMEM with Kenward-Roger's method for degrees of freedom
3. Average left and right sides for whisker speeds and positions, calculate asymmetry
4. Convert locomotion speed to meters/second

5. LMEM with Kenward-Roger's method for degrees of freedom
6. Report whisker mean angular position, amplitude, spread, asymmetry, protraction, retraction and locomotion speeds. If using rats, exclude locomotion speed
7. Provide graphs with per animal data points and bars showing clip means
8. Provide example whisker traces, attach video clips used for those
9. Provide example video stills
10. Report presence or absence of robust contact-related behaviours
11. Report differences between treatment groups, sexes, ages
12. Report all details required by ARRIVE guidelines (Kilkenny et al., 2010; Sert et al., 2020). Follow the FAIR guidelines on data management and sharing (Wilkinson et al., 2016).

ii) Automation

I have used a semi-automated whisker tracker (ARTv2) throughout the thesis. I have replaced qualitative scoring of spread reduction and whisking with quantitative (PC-DC) spread measure, PC whisking amplitude and PC mean angular position. This results in a more streamlined process of whisker tracking and analysis, as well as being more quantitative and objective. Some manual steps remain in the process, including i) capturing the clips by the trigger; ii) reviewing clips to fit selection criteria, and iii) cutting the clips into PC and DC segments. There are ways to configure the camera to automatically trigger using a percentage of pixel changes that might be worth exploring in the future. With whiskers being so lightly coloured and small, it is not yet possible to automatically identify the first whisker touch with an object to split the clip into PC and DC segments; however, this might become a possibility with the development of new computing techniques. However, as I am always blinded to the treatment group of

the animal, any manual step, such as reviewing the tracking and trimming the clips, is not biased.

iii) Placing whisker movements within the context of other tests

In Chapter 5, I installed an overhead infrared camera to monitor the position of the mice throughout the whole trial. This gave a lot of insight into their behaviour, compared to my usual data collection, which only collects snippets of videos of ~1 second, rather than a full ~10-minute video for each trial. The reeler mice spent the same amount of time around the object, compared to the wildtype mice. And despite often being further from the object, this was not a statistically significant finding. The addition of the overhead camera provides a useful extra monitoring system alongside the high-speed video footage. It can also offer flexibility, allowing researchers to collect additional data after the trials have been conducted, such as the position of the mouse in the arena, their proximity to the object, or number of interactions with the object. This camera is more in-line with other protocols that constantly monitor throughout open field or novel object tasks. It can provide validation against other locomotion measures and an even better quality one would also allow me to easily record grooming, rearing and other similar behaviours.

I have shown here that the protocol can be easily extended to include a sequential object task and a habituation study. Indeed, the protocol is adaptable, and I believe that it is fairly easy to incorporate other tests, including social tasks, gap crossing and balance tasks. The sequential object exploration task is probably the most comparable to the standard novel object recognition (NOR) task, one of the gold-standard behavioural tests. According to the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS, Young et al., 2009), out of all the preclinical rodent tasks they suggest are translatable to human symptoms, only NOR and social tasks rely on innate behaviour, while all other cognitive tasks require training and motivation by food or aversion. Young et al. (2009) suggest that NOR is not a goal-oriented task, presumably because there is no training and no “goal” of solving a problem. In contrast, whisker tracking allows

precise measurement of whisker angles and speeds related to object exploration (Gillespie et al., 2019), which could be considered a natural, goal-oriented behaviour for the rodent, if the rodents' goal is to familiarize themselves with their environment, including the objects. It has been shown several times (Arkley et al., 2014; Grant et al., 2016, 2018; Simanaviciute et al., 2022) that whisker tracking during a novel object exploration (NOE, not recognition) task without any training or habituation can also assess attention without relying on external motivation. The work presented here shows that the sequential object exploration task can also be used for the same purpose and that the addition of a second object, as compared to NOE task, allows to assess attention in more detail depending on the choice of objects. It might be beneficial to combine whisker tracking with the classic NOR task, to study the object contact in more detail and to incorporate the memory aspect when assessing these measures in novel and familiar object.

With both NOR and social tasks suggested as being translatable to humans (MATRICS, Young et al., 2009), they are perhaps the next priority tasks to develop alongside my whisker measurement protocol (Figure 6-1). Indeed, in reeler mice, as part of my future work, I have already attempted integrating a NOR task with whisker tracking on a visit to University of Goettingen, Germany. I found that I could only image one object at once with one high-speed video camera, although two objects were presented to the animal simultaneously. I have not analysed this data yet, but it will be interesting to see whether there are any differences between the reeler and control mice.

Another recommendation for future studies is to test rodents in a whisker-related social task to better understand that behaviour. In Potter et al. (2023), Poly I:C treatment positively affected social interactions where adult female MIA rats had increased sniffing and following of conspecifics, thus, this model might be useful to further investigate. In fact, I have started to develop a social task (Figure 6-2) in the NRXN1 mouse model of autism spectrum disorders, whose whisker-related behaviour has not been previously described. Within my usual experimental arena, I used two barred containers with two unfamiliar mice (one wildtype and one transgenic). A parallel study was run as an additional control, with one familiar and

one unfamiliar mouse in the containers. Control and transgenic mice were introduced to the arena in both cases, and I will soon start to track the whiskers of these focal mouse as they interact with the sex-matched mice in the containers. This is one of the first social whisker tasks, and it will be interesting to see the results that I have collected during my visit to Dalhousie University, Halifax, Canada.

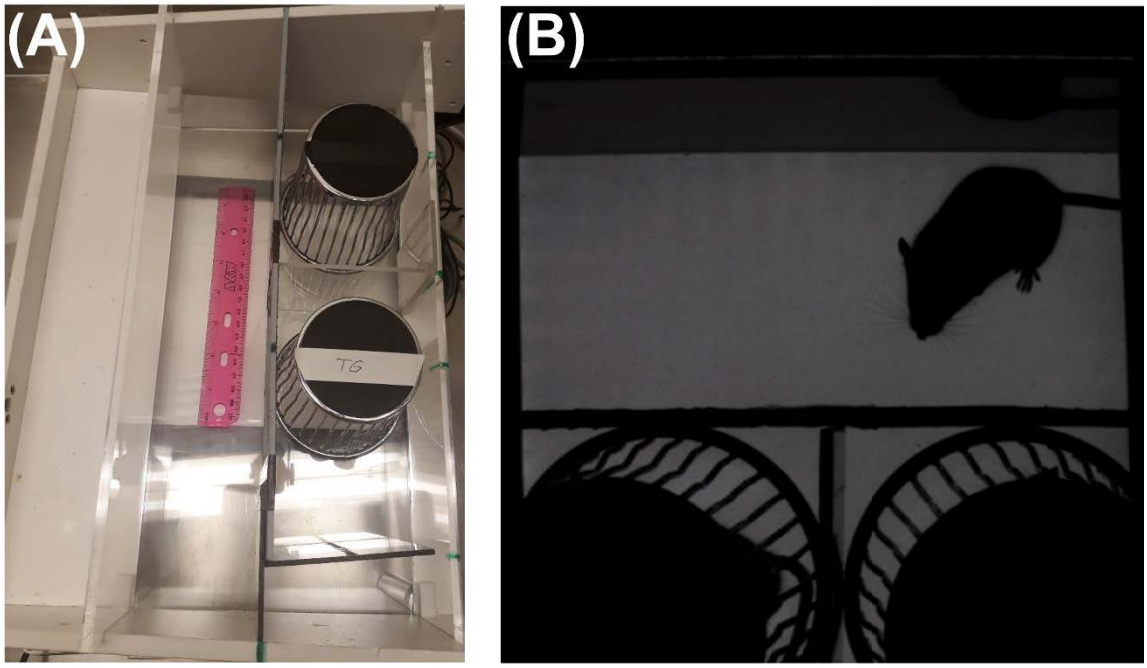


Figure 6-2 **The social task set up.** Demonstrating the barred containers and the arena in A, as well as a video still from the high-speed video camera in B.

Addressing thesis questions

From my Literature Review (Chapter 2), I identified some questions for my thesis to address, including:

i) How can we move towards a standard test of whisker movements?

The protocol I have presented here is a first attempt at moving towards a standard test for testing whisker movements. It will continue to expand and evolve from here.

ii) *We do not always see differences in whisker movements, so which models would I recommend to study?*

This is perhaps a more complex question. In my thesis, I focussed on models in which the standard behavioural tasks do not have clear, reproducible results, and these might be good ones to continue to focus on. Indeed, with the cost of the high-speed camera and lighting, and the time investment of video tracking in a new software, it is probably best to reserve whisker measurements for models where the behavioural phenotype is relatively subtle or nuanced. Additionally, these types of models and instances are also probably a good fit for applying whisker movements:

- When the speed of testing is important, such as in treatment screening studies;
- Where it would be useful to check that animals adequately acquired contact-related information, such as in combination with object memory tests;
- Where reproducibility between laboratories is especially important. Because of the robust contact-related changes in whisker angles and speeds, this is a good method to check for experimenter or laboratory differences before proceeding with other tests.

Even if differences in whisker movements are not observed, amendment of the protocol to include other tasks, such as sequential object, habituation, NOR and social tasks, might be an important step in making whisker movement measurements more applicable to a wider range of rodent models.

iii) How does whisker movement fit with other behavioural tests?

Results from my thesis suggest that the protocol can fit very well as an add-on to most batteries of tests, due to being non-invasive and quick. It is best being done as the first test when animals are naïve to the environment, which is complementary to most batteries of tests where animals must be habituated or trained. The whisker tracking study could even be conducted as part of the

habituation period for other studies, such as in the novel object recognition paradigm.

In the future, whisker tracking could potentially be integrated simultaneously with home-cage monitoring or other observational studies of naturalistic behaviours that do not require training such as general locomoting, interacting with objects or conspecifics, feeding and sniffing behaviours. It may also be possible to combine the protocol with other emerging quantitative metrics, such as measuring ultrasonic vocalisations when studying communication of fear, anxiety, or social and maternal behaviour.

While I demonstrate here that the usual whisker task can be adapted to include sequential object and social tasks, it may be more challenging to integrate measuring whisker movements within another task that requires strict training regimes and does not combine well with environmental variation, such as when incorporating the lightbox and cameras. I have attempted to integrate the protocol with a novel texture recognition task in the reeler mouse model and was able to successfully collect video clips for whisker tracking. However, animals were not able to perform the texture recognition task, and it is unclear if that was due to intrinsic differences or due to the environmental effects like additional handling or noise caused by the attempted integration of the two tests.

6.2 Limitations and future work

Above, I have discussed the importance of developing a social and NOR version of the whisker measurement task as an add-on to the usual protocol. These tasks are useful as they are thought to be more translatable to humans (MATRICS, Young et al., 2009) than other behavioural tests. Indeed, one of the drawbacks of the whisker task, which may have limited its uptake, is the challenge associated with translating findings to humans. Nevertheless, we do need better behavioural tasks for rodents too, and this will help us to better understand neurodegenerative disease in humans as well.

Overall, measuring whiskers in rodent models of disease has mainly been done by researchers in our laboratory, visiting several laboratories around the world. However, other people have measured whiskers for the assessment of facial palsy recovery (Miura et al., 2023), suggesting that other labs can easily adopt it.

There were some limitations to my experimental studies, that I have identified as I went along, these include:

Chapter 3

- Sample size of transgenic mice at 3 months was low
- It was not a longitudinal study; however, that means no habituation effects
- It was a female-only study

Chapter 4

- Could not measure locomotion speed
- There were no different time points
- Addition of object textures complicated the study design

Chapter 5

- Variable ages and sexes due to increased mortality in reeler mice
- Reeler mice did not touch the object as much which reduced video clip numbers in the usual object exploration task

Indeed, from my work I can see several limitations that need to be considered when using the whisker measurement protocol:

i) The sample size can be limiting, which may include the animals themselves or clips not passing the criteria, because younger 3xTg animals behaved differently (Chapter 3) or object was not touched as often (Chapter 5). 312 videos were collected in Chapter 3 mouse study and 183 of them made it to analysis. 629 videos were collected in Chapter 4 rat study and 114 of them made it to analysis. 403 videos (naïve and previously exposed mice) were collected in Chapter 5 novel object exploration study and 73 (naïve mice only) of them made it to

analysis. That is 41% for Chapter 3 and 82% video loss rate for both Chapters 4 and 5. This video loss rate is not due to the ARTv2 tracker, but mostly due to the video selection criteria, especially the requirement to only include videos with suitable PC and DC sections. In part, this limitation was also due to the travelling nature of these studies, as I could not go back to get more suitable clips. In the future, laboratories which adopt this method within their animal facilities would have their own set ups and would be able to access the animals multiple times to get more samples when needed.

ii) There may be differences between individuals. Differences between wildtype animals were detected in Chapter 3 and could have been related to age effects, or other environmental considerations related to previous experiences, as those were not the same animals tested at a later age but rather another group of animals. However, as using this method very quick data collection, I suggest those differences are unlikely to be due to my experimental set up and day-to-day variation.

iii) Different disease symptoms make it difficult to collect appropriate animal groups, e.g., mortality rate in reeler mice and in later stages of Alzheimer's models, so age-related changes or disease progression with time might be harder to study in longitudinal studies. It is also important to have those bigger gaps of time in between testing of the same animals, as habituation was found to influence whisker movements.

iv) Translatability - whisker movement deficits can be difficult to translate to human disease symptoms which is why I did not put the findings of Alzheimer's study into the context of human AD.

v) Habituation affects whisker movements, so we need to work out when to do repeat testing. Perhaps a large gap is needed (e.g. in the SOD1) or use different animals at different ages (e.g. 5xFAD, HD etc).

There are also the standard animal differences that need to be considered, including:

vi) Age differences - we do not know how whisker movements vary over age;

vii) Strain differences have been identified before (Simanaviciute et al. 2020);

viii) Sex differences - I tested both sexes and observed differences in MIA rats, but not reeler mice;

ix) Visual deprivation - mice deprived of vision since birth rely even more on their whiskers: those were found to be longer and thicker than in control mice; additionally, the visually deprived mice had an enlarged barrel field (Rauschecker et al., 1992). In fact, Grant et al. (2018b) showed that female 5xFAD mice with retinal degeneration (rd) had lower mean angular positions during object contact. During sequential object exploration females with rd had higher whisker retraction speeds in tunnel running. This shows that measuring whisker movements can quantify the effects of rd during exploratory behaviour in these mice. None of the animals tested were visually deprived and thus this was not a consideration for the findings in this thesis. However, it can be a factor in other studies;

x) Individual differences are likely to also occur, although these have not been properly investigated before. There are likely to be individual differences in disease manifestation, too. Lorusso et al. (2022) recommended grouping or clustering individuals according to the severity of their disease symptoms, which might be a good way to proceed, rather than simply dividing animals into treatment and control groups;

xi) Litter effects have not been tested for in whisker parameters and thus it is not clear if they would be a significant factor.

xii) While not all studies may want to track whiskers, they should measure and report whisker health (Kahnau et al., 2022). Barbering is important in social and exploratory behaviours and has even been suggested as a valid model of trichotillomania by Garner et al. (2004).

Another theme that came about from my Literature Review was reproducibility, which my thesis has not addressed so far. However,

1) Locomotion and whisker movements of two sets of SOD1 mice were found to be affected by 120 days old.
2) While I did not perform any reproducibility studies, robust contact-related behaviours are seen in all models, indicating that it is likely to be reproducible in all wildtypes, but may also have subtle strain, age and sex differences.

Generally, the protocol is set up to include as little variation as possible, especially compared to other tests available. There are multiple tests that could be conducted in future work to address various levels of reproducibility, including running the same experiments in the same or different laboratories, with the same or different experimenters, and with the same or different animals. For example, an interesting experiment would be to study the 3xTg-AD mouse model in several different laboratories following the same protocol, which would help to establish how reproducible the method is. It may also shine a light on the main strength and weakness of this method which is that this was always done by visiting other facilities. Someone else always had to be there to handle the animals while running the experiments – would the results be different if only one person was handling and running the experiments, especially if animals tested are familiar with the handler but not the visitor? Additionally, I was the only person to analyse the whisker parameter data in this thesis – will the protocol be robust and clear enough for another person to analyse it the same way? The take up of this method by other laboratories may equally increase or reduce its reproducibility.

Future work in whiskers - what is now possible?

There have been many recent advances in naturalistic behaviour tracking and its combination with neuronal recordings, as discussed in the Literature Review (Chapter 2); however, this has not yet been related to whisking, despite it being essential to rodent's survival and having major impact on the locomotion measures that are often estimated from pose in these methods. Wiltschko et al. (2015)

suggests that whisker tracking should be incorporated with pose estimation to fully capture the behaviour.

Home cage systems (Endo et al., 2011) also provide a way to observe the animals non-invasively in their home environment. However, they don‘t often give the same level of fine-scale movement and quantification of behaviour as whisker movement measures. Despite this consideration, they are constantly and consistently being developed and may be an interesting avenue of future research. Indeed, to increase reproducibility, it is important to automate as much as possible, make tests quick and reduce day-to-day variability. Including general activity and behavioural measures over longer periods of time, such as home-cage monitoring, might also be useful.

In my own work I have seen how whisking differs between different “control” animals. Often control animals are matched to the disease model, and thus can come from different background strains, different breeders, or are tested in different laboratories on separate occasions (Simanaviciute et al., 2020). There does not seem to be one solution to this crisis, however, one recommendation is to use home-cage monitoring to record long-term behaviour and perform some testing in the home-cage environment.

Throughout the chapters of this thesis, PC amplitude, PC and (PC-DC) mean angular position, PC and (PC-DC) spread and (PC-DC) asymmetry were affected the most. However, all whisker measurements except for mean angular position are needed to study the robust contact-induced changes. In earlier studies such as Simanaviciute et al. (2020), which included 9 different mouse models, all whisker parameters were affected. Overall, the measurements taken are the same in all disease models, but different deficits may show up as disease manifests and thus it may be beneficial to continue measure all whisker parameters. For these reasons, I recommend continuing studying all whisker parameters until more research is conducted on the validity of the individual whisker parameters. A newer approach to data analysis may be to use machine learning approaches to cluster disease phenotypes (Lorusso et al., 2022) and to estimate poses from

deep learning approaches, such as LEAP software as used in Klibaite et al., (2022). Despite being available for more than a decade now, these have never been tested in whisker tracking studies before and would initially take longer to train and validate compared to ARTv2 software, but in the end might result in needing less manual validation from the experimenter. Such an approach might also help to keep whisker identities during tracking, which would provide more detailed, individual whisker data for analysis.

Recommendations for behavioural tasks

Overall, I recommend that all behavioural tasks for rodents should be:

Quick

- Reducing handling time
- Reducing time away from home-cage
- Reducing duration of the experiment

Objective

- Reducing experimenter bias
- Blinding reviewer to the animal's condition
- Automated, reducing all manual steps

Highly granular

- Not just simple counts, scores or durations

Maximising sample sizes

- Allow recording the same animal over time
- Easy to integrate with other tasks
- Use analysis approach that automatically determines degrees of freedom

Incorporating both sexes

Adaptable, allowing tailoring to the animals studied

- Open field for motor and object tasks for cognition and sensing as the standard
- Monitor general behaviour constantly with overhead camera
- Bespoke additions of tasks
    - Change type of object
    - Sequential object task
    - Habituation study
    - Social study

I propose that measuring whisker movements addresses all these points, and with greater uptake from other labs, has the potential to improve rodent welfare during behavioural testing, especially by refining tasks to make them non-invasive, reducing the need for rodent training, making sure tasks are quick and providing quantitative data. Throughout my thesis, I present whisker movement measurement as a powerful tool able to reveal treatment and sex effects in three rodent models with challenging behavioural phenotypes. I have streamlined the whisker measurement protocol, which has increased standardisation and automation. I think future research measuring whiskers in rodent models of neurological disease should focus on developing the protocol alongside NOR and socials tasks, an area that I have already started to work on.

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ORIGINAL ARTICLE

Genes, Brain and Behavior

Abnormal whisker movements in the 3xTg-AD mouse model of Alzheimer's disease

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Abstract

Alzheimer's disease is the most frequent form of dementia in elderly people. The triple transgenic (3xTg-AD) mouse model of Alzheimer's Disease is important in biomedical research as these mice develop both neuropathological and behavioural phenotypes. However, their behavioural phenotype is variable, with findings depending on the specific task, as well as the age and sex of the mice. Whisker movements show motor, sensory and cognitive deficits in mouse models of neurodegenerative disease. Therefore, we examined whisker movements in 3, 12.5 and 17-month-old female 3xTg-AD mice and their B6129S/F2 wildtype controls. Mice were filmed using a high-speed video camera (500 fps) in an open arena during a novel object exploration task. Genotype and age differences were found in mice exploring the arena prior to object contact. Prior to whisker contact, the 3-month-old 3xTg-AD mice had smaller whisker angles compared with the wildtype controls, suggesting an early motor phenotype in these mice. Pre-contact mean angular position at 3 months and whisking amplitude at 17 months of age differed between the 3xTg-AD and wildtype mice. During object contact 3xTg-AD mice did not reduce whisker spread as frequently as the wildtype mice at 12.5 and 17 months, which may suggest sensory or attentional deficits. We show that whisker movements are a powerful behavioural measurement tool for capturing behavioural deficits in mouse models that show complex phenotypes, such as the 3xTg-AD mouse model.

KEYWORDS
Alzheimer's, animal behaviour, disease model, mouse model, neurodegeneration, rodent, sensorimotor, transgenic, vibrissae, whisker

1 | INTRODUCTION

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disorder, the most frequent form of dementia in elderly people.[1–3] Mouse models are essential for improving our understanding of the neural and behavioural changes that occur during AD progression, and to develop novel therapeutic targets.[4,5] The triple transgenic (3xTg-AD) mouse model is considered to have high validity as these mice develop both Aβ plaques and tau tangles,[6] as well as show cognitive deficits.[7,8] The 3xTg-AD mice have altered performance on sensory tasks involving vision,[9] olfaction,[10] and touch,[11] as well as motor[12,13] and cognitive

tasks.[5,14–16] The 3xTg-AD mice generally perform worse than their wildtype controls in spatial learning and memory tests.[16,17] They have a complex motor phenotype and have even shown an enhanced motor phenotype at 6 and 16 months of age.[12,13] They show higher frailty measures[18] and have a shorter lifespan than their wildtype background strain. Male 3xTg-AD mice also have a shorter lifespan than females,[19] as well as altered immune function and gene expression.[20]

Behavioural studies have shown quite variable outcomes with these mice, especially in motor and cognitive tasks, such as spatial learning and memory.[12,16] Age, sex, experimental apparatus and test design all impact the performance of 3xTg-AD mice during behavioural tasks.[16,18,21] Therefore, a better understanding of the behavioural manifestations that occur in this model of AD is needed. Measuring whisker movements in mouse models has been suggested as an easy and robust way to capture elements of sensory, motor and cognitive deficits in mice.[11] Such deficits have been shown in mouse models of Amyotrophic Lateral Sclerosis,[22] Huntington's Disease,[23] anxiety,[24] Alzheimer's Disease,[11,25] as well as Cerebellar Ataxia, Somatosensory Cortex Development disorders and Ischemic stroke.[11]

Rodents rely on their whiskers as their primary sense of touch.[26] In addition to their sensory function, whisker movements indicate aspects of motor control; they can move rhythmically to-and-fro in a process called whisking, which occurs up to 25 Hz in mice.[27] Mice also precisely control their whisker movements during object exploration.[28–30] When a mouse contacts an object with their whiskers, they tend to decrease their whisker angles (the angle between the head and whiskers), reduce whisker spread, increase whisker asymmetry and amplitude, and slow whisker speeds.[29–31] These changes allow many whiskers to contact a surface for longer durations, hence increasing the quality of sensory information from whisker contacts.[30] The positioning and focussing of many whiskers onto an object are thought to be associated with attention.[32,33] These whisker movements are disrupted in many mouse models of disease and may be indicative of sensory, motor or cognitive deficits.[11,22,23]

We have previously shown that 17-month-old female 3xTg-AD mice had smaller whisker angular positions and retraction speeds compared with wildtype controls when moving around their environment without object contacts.[11] However, it is important to measure these changes at different age points and during object contact to better understand the deficits in whisker movements in this mouse model. Therefore, the aim of this study is to investigate whisker movements in the 3xTg-AD mouse model at different ages, before and during object exploration. This study was designed based on the recommendations of Simanaviciute et al.[11] as shown in Figure 1. To detect sensory, motor, and/or cognitive deficits in the 3xTg-AD mouse model, we tested for all the suggested steps in Figure 1. We scored whisker movements prior to object contact and during object exploration using both qualitative and quantitative measures in order to detect any deficits in whisking behaviour in the 3xTg-AD mice.

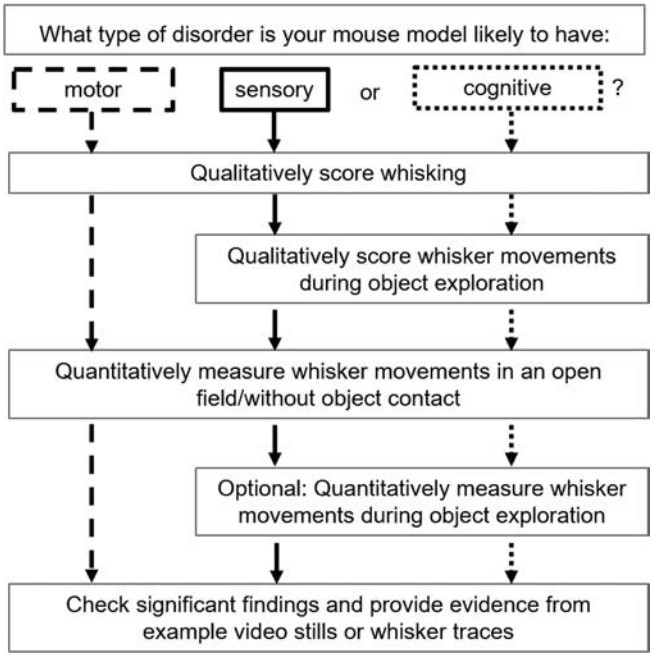

FIGURE 1 Methods schematic for testing mouse models with likely motor, sensory or cognitive symptoms, suggested by our previous research (adapted from Simanaviciute et al.[11]).

2 | MATERIALS AND METHODS

2.1 | Animals

A total of 38 female mice were used in this cross-sectional study: 17 transgenic (3xTg-AD, JAX # 004807) mice (3 at 3 months, 6 at 12.5 months, 8 at 17 months) and 21 wildtype (B6129S/F2 WT, JAX# 101045) mice (8 at 3 months, 7 at 12.5 months, 6 at 17 months). All mice were born in-house at Dalhousie University from breeding pairs purchased from Jackson Laboratory (Bar Harbour, Maine USA). The 3xTg-AD mice were engineered by injecting APPSwe and tauP301L transgenes into single-cell embryos of homozygous PS1M146V knock-in mice. This causes Aβ42 aggregation in the frontal cortex and the hippocampus at around 3 months of age, extracellular plaques in the frontal cortex and the hippocampus at 6 months of age, and hyperphosphorylated tau tangles at 12 months of age.[6] Our study spans these changes by observing mice from 3 to 17 months of age. Due to increased mortality in male mice by 17 months of age,[19] only female mice were included in this study.

Mice were weaned at 21 days of age, their ears were punched for individual identification, and they were housed in same sex groups of 2–4 in 30 × 18 × 12 cm translucent polycarbonate cages with wire lids and microisolator tops. Cages contained woodchip bedding (Fresh Bed, Shaw Resources, NS, Canada) and a 4 × 7 cm PVC tube for enrichment. They were kept in a climate controlled (22°C ± 2°C) vivarium on a reversed 12:12 light: dark cycle with lights off at 09:45 am. All behavioural testing was completed during

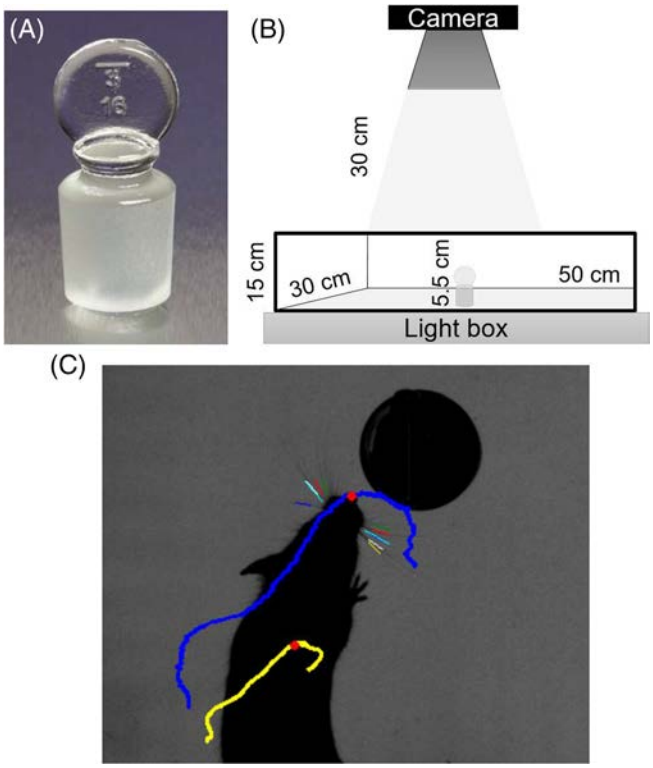

FIGURE 2 Data collection and video analysis. Panel A shows the glass bottle stopper object used in the experiments; Panel B illustrates the filming set-up, the object size and location in relation to the Perspex box, and the distance between the arena and high-speed video camera. The field of view in light grey corresponds to the video still in c) showing an example video clip. ARTv2 LocoWhisk software was used to automatically locate the mouse centroid (red point, yellow line), nose tip (red point, blue line) and whiskers (coloured lines), and detects them on a frame-by-frame basis.

the dark (active) portion of the light: dark cycle. Mice had ad libitum access to Purina Laboratory Rodent Chow #5001 (Agribrand Purina, Strathroy, Ont., Canada) and tap water. Mice were treated in accordance with the regulations set forth by the Canadian Council on Animal Care and the experimental protocol was approved by the Dalhousie University Committee on Animal Care and the local ethics committee at Manchester Metropolitan University.

2.2 | Experimental procedures

For filming whisker movements, mice were placed in a transparent Perspex rectangular arena (30 × 50 × 15 cm) which was lit from below by an infra-red light box (LEDW-BL-400/200-SLLUB-Q-1R-24 V, PHLOX) (Figure 2B). Mice were filmed from above using a digital high-speed video camera (Phantom Miro ex2) recording at 500 frames per second with a shutter-speed of 1 ms and resolution of 640 × 480 pixels. A Pyrex glass bottle stopper (Figure 2A) was placed inside the arena as an object to explore. Multiple 1.6 s video clips (800 frames) were collected opportunistically (by manual trigger) when the mouse moved into the camera's field of view.

2.3 | Video analysis: qualitative whisker scores

Video clips were selected for analysis based on the criteria developed by Grant et al.[22] These criteria were: (i) the mouse was clearly in the frame; (ii) both sides of the face were visible; and (iii) the head was level with the floor (no extreme pitch or yaw). In these clips, *whisking* by mice was scored on a four-point scale from no whisking (0), to only retractions (1), only protractions (2) or both retractions and protractions (3). To qualitatively assess whisker behaviours and exploratory strategies, all of the video clips that met the above criteria were scored based on a system developed by Grant et al.,[31,34] in which *contact-induced asymmetry*, *spread reduction*, and *head turning asymmetry* were measured.[27,35] When the mouse was contacting an object with their whiskers, *contact-induced asymmetry* (CIA) was scored on a three-point scale from absent (0), to showing increased contralateral protraction (1), reduced ipsilateral protraction (2) and both increased contralateral protraction and reduced ipsilateral protraction (3). Object-directed whisker *spread reduction* was scored as absent (0) or present (1) when whisker spread decreased following object contact. *Head turning asymmetry (HTA)* was scored as present (1) or absent (0), during a head turn.

2.4 | Video analysis: quantitative analysis of locomotion and whisker movements

For quantitative analysis of whisker movements and locomotion, video clips were divided into pre-contact (PC) and during object contact (DC). Therefore, the clip selection criteria were amended to also include (i) the mouse must be travelling towards the object in the PC section of the clip; and (ii) the whiskers were only contacting the object and not the vertical arena walls, in the DC section of the clip. In this way, general whisker movements could be assessed for motor behaviour in the PC section of the clip (similar to an open field), and object exploration could be assessed in the DC section of the clip. Only clips that had both considerable PC and DC segments (>0.2 s) were included in this quantitative analysis. The clips were tracked using the Automated Rodent Tracker, version 2 (ARTv2).[36] This used image processing to automatically locate the snout and the centroid of the mouse, for *locomotion speed* calculations (from the yellow trace in Figure 2C). A ruler was filmed at the start of each episode of data collection to enable a calibrated measure of locomotion speed in metres per second.

The whisker detector program (ARTv2) found the orientation and position of the snout, and the whisker angles (relative to the midline of the head) of each identified whisker (Figure 2C). The ARTv2 program is only able to detect whiskers and does not maintain the identity of the whisker between frames (i.e., tracking); rather, a mean angle is calculated from each frame using all detected whiskers. Larger whisker angles represent more forward-positioned whiskers. If a whisker is occluded (such as by whisker crossing) the software will not detect it; therefore, the number of whiskers detected can vary from frame to frame, with a total of

2–12 whiskers detected in each frame (with around 10–12 whiskers being usual, 5–6 on each side). Whisker detection was validated by manually inspecting the software annotations overlaid onto the video frames. From 1 to 12 video clips per mouse were included in data analysis (Supplementary Table 1), resulting in a total of 183 whole clips, all of which contained both PC and DC sections. PC sections ranged from 100 to 600 frames per clip, whereas DC sections ranged from 100 to 625 frames.

Mean whisker angle was calculated by taking the mean of all the detected whiskers on each side, on a frame-by-frame basis (Figure 2C). The following variables were then calculated from the mean whisker angles: *mean angular position* (the average whisker angle), *amplitude* (2√2* the standard deviation of whisker angles, to approximate the range of whisker movements), *asymmetry* (the difference in whisker angles between the left and right sides), and the *mean angular retraction* and *protraction speeds* (calculated as the average speed of all the backward (negative) and forward (positive) whisker movements, respectively). For the first time in a mouse model study, whisker spread was also quantified. Mean angular position and spread are considered the two most informative parameters to assess in whisking,[30] thus this was an important quantitative measure to supplement the qualitative scoring of spread reduction. *Spread* was scored as the standard deviation of all tracked whisker angular positions. For mean angular position, amplitude, whisker speed and spread, the mean values for right and left whisker measurements were used to give one value per video clip.

2.5 | Statistical analyses

For all qualitative and quantitative whisker measurements, each variable was compared between wildtype and 3xTg-AD mouse, at each age (3, 12.5, and 17 months). Qualitative scores of whisking behaviours were analysed using the Kruskal–Wallis test with Dunn's post-hoc tests using GraphPad Prism 8 software, as these were on ordinal scales and not normally distributed.

Quantitative measures of the pre-contact (PC) whisker variables were first analysed. Then, the changes in whisker measurements during object exploration were analysed by subtracting the during-contact measures from the pre-contact measures (PC-DC). PC-DC was chosen, rather than DC-PC, as it is more intuitive to identify increases in variables during contact as positive, and reductions as negative; in addition, many of the whisking parameters were expected to be higher in PC. A Linear Mixed-Effects Model was constructed using the package lme4[37] in R Studio to analyse the effect of age and genotype on all PC and PC-DC whisker variables. The model computed *F* tests on the fixed effects of age and genotype and provided *p*-values using a type III ANOVA, as well as interaction effects (although all the interaction effects were not significant and will not be referred to further in the main text, though, see Supplementary Tables 3 and 4 for more detail). Since the mice were filmed repeatedly exploring an object, and every subsequent video clip was different, with the mouse acquiring increasingly more information, each video clip was treated as a within variable, but the degrees of freedom and F-statistics were approximated using a Kenward–Rodger's method.[38] This method takes account of uneven and low sample numbers (such as from the 3-month-old animals). The degrees of freedom were automatically determined to be anywhere between the number of animals and the number of video clips for each particular measurement analysed. A significance value of $p < 0.05$ was used throughout. Significant pairwise comparison results are indicated on all figures with an asterisk (*). The Kenward–Rodger's approximation is the preferred method of approximating degrees of freedom over Satterthwaite's method,[39,40] and of reporting *p*-values over likelihood ratios and Wald *t*-values.[41] It also produces acceptable Type 1 error rates in smaller sample sizes in models fitted with restricted maximum likelihood.[41] We also conducted Satterthwaite's method to approximate *F*-tests and degrees of freedom on the quantitative measures. Significant results identified from this method were less conservative than those calculated by the Kenward–Rodger's approach, therefore, increasing the confidence in our statistical reporting.

3 | RESULTS

3.1 | Qualitative whisker behaviour

The whisking scores from the qualitative measures show that, while all wildtype mice whisked, with median of 3, the 3xTg-AD mice had lower scores with medians of 2–3 ($H [5, 183] = 39.9$, $p < 0.001$; Figure 3A). At 12.5 months ($p = 0.008$) and 17 months ($p < 0.001$) of age the 3xTg-AD mice had significantly reduced whisking scores compared with the age-matched wild types, showing more whisking movements which were only protractions in the 3xTg-AD mice, rather than the protractions and retractions associated with whisking in the wildtype mice. The whisking scores of the 17-month-old 3xTg-AD mice were also significantly lower than those of the 3-month-old 3xTg-AD mice ($p = 0.020$). There were no significant differences in HTA scores between 3xTg-AD and wildtype mice ($H [5, 101] = 6.74$, $p = 0.241$, Figure 3B). During object exploration there were significant differences in spread reduction ($H [5, 183] = 20.6$, $p < 0.001$) and CIA ($H [5, 183] = 26.4$, $p < 0.001$) between 3xTg-AD and wildtype mice. The 12.5-month-old 3xTg-AD mice had significantly lower whisker spread reduction values than their wildtype controls ($p = 0.008$), and these were also lower than the values for 3-month ($p = 0.003$) and 17-month ($p = 0.002$) 3xTg-AD mice (Figure 3C). The CIA scores of the 3-month-old wildtype mice were significantly higher than the age-matched 3xTg-AD mice ($p = 0.008$) and the 12.5-month wildtype mice ($p < 0.001$, Figure 3D). Detailed statistical information for every comparison in qualitative analyses can be found in Supplementary Table 2.

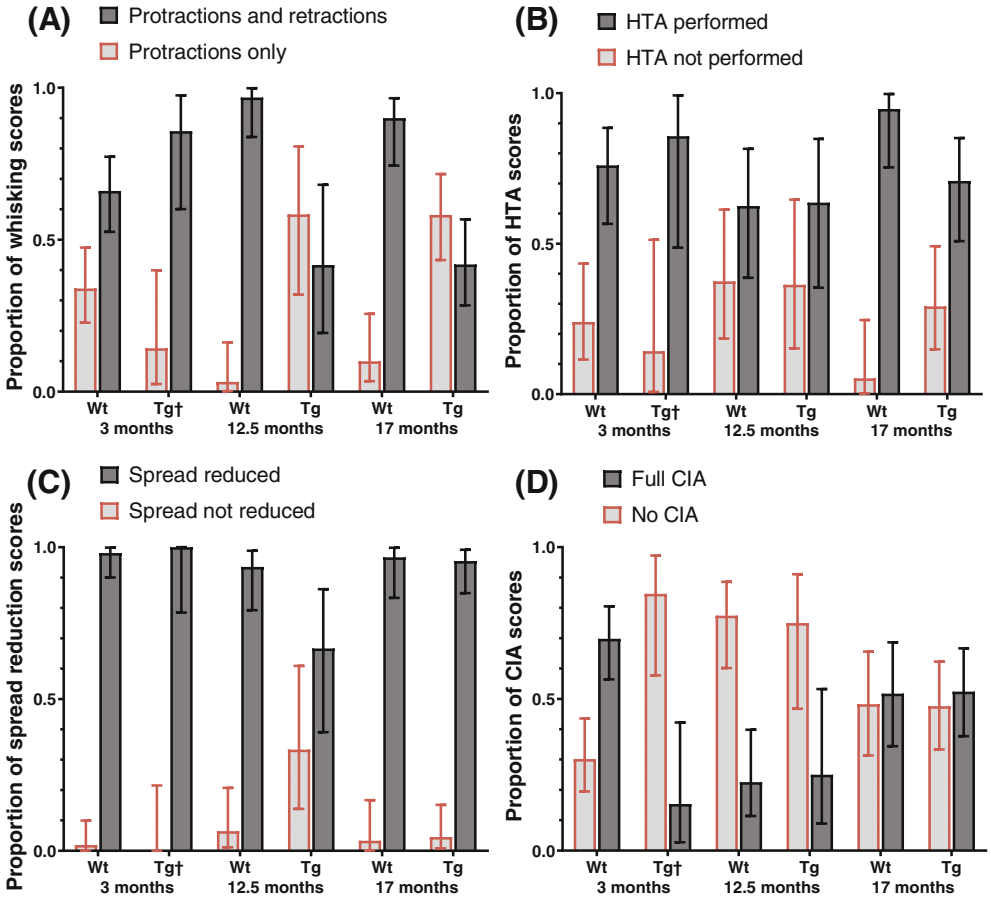

FIGURE 3 Qualitative whisker behaviour scores for (A) whisking, (B) head-turning asymmetry (HTA), (C) spread reduction, (D) contact-induced asymmetry (CIA). The bars indicate the proportion of clips where the behaviour occurred or did not occur, with confidence intervals. † indicates $n = 3$ mice.

3.2 | Pre-contact (PC) quantitative whisker and locomotion movements

For pre-contact whisker amplitude, there were significant main effects of both genotype (F [1, 29.36] = 12.43, p = 0.001) and age (F [2, 27.08] = 4.06, p = 0.029). Specifically, pre-contact whisker amplitude was lower in 3xTg-AD mice than in the age-matched wildtype mice (Figure 4A). Pairwise tests show that these differences were significant in 17-month-old mice (p = 0.013). These differences can also be seen in the pre-contact whisker traces in Figure 6. Furthermore, there was a difference in pre-contact whisker amplitude between 3 and 17-month wildtype mice (p = 0.042) as whisker amplitude increased with age.

For the pre-contact whisker angular position, there were significant main effects of genotype (F [1, 32.82] = 20.38, p < 0.001) and age (F [2, 32.66] = 6.96, p = 0.003). The pre-contact whisker angular position was consistently lower in the 3xTg-AD mice compared with the wildtype mice (Figure 4B), especially at 3 months of age (p = 0.040). These results are supported by the video stills (Figure 5) and the whisker traces (Figure 6), where pre-contact mean whisker angles were lower in the 3xTg-AD mice than the wildtype mice. Wildtype mice at 3 months of age also had larger pre-contact mean angular positions than wildtype mice at 12.5 months (p = 0.038) and 17 months of age (p = 0.006).

In the pre-contact whisker spread, there were significant main effects of genotype (F [1, 32.83] = 10.62, p = 0.003) and age (F 2, [32.67] = 5.61, p = 0.008). However, pairwise tests did not show any significant differences (Figure 4C). There were no significant differences in pre-contact whisker movements in locomotion speed, asymmetry, retraction speed and protraction speed (Supplementary Figure 1). Detailed statistical information for every comparison in PC quantitative analyses can be found in Supplementary Table 3.

3.3 | Contact-related (PC-DC) quantitative whisker and locomotor movements

Both wildtype and 3xTg-AD mice showed robust changes in whisker movements in response to object contact at all ages as indicated by a reduction in locomotion speed (Supplementary Figure 1A), retraction and protraction speeds (Supplementary Figure 1C and D), and an increase in whisker asymmetry (Supplementary Figure 1B) and amplitude (Figure 4A) following an object contact (PC-DC). The whisker traces (Figure 6) show this increase in asymmetry as the left (red) and right (blue) traces separate following object contact in all examples. Since these behaviours were robust in all mice, there were no significant effects of genotype or age in the contact-related (PC-DC) variables of whisker amplitude (Figure 4A), whisker angular position (Figure 4B), locomotion speed, whisker asymmetry, retraction speed and protraction speed (all ps >0.05, Supplementary Figure 1A-D). However, in (PC-DC) whisker spread, there were significant main effects of

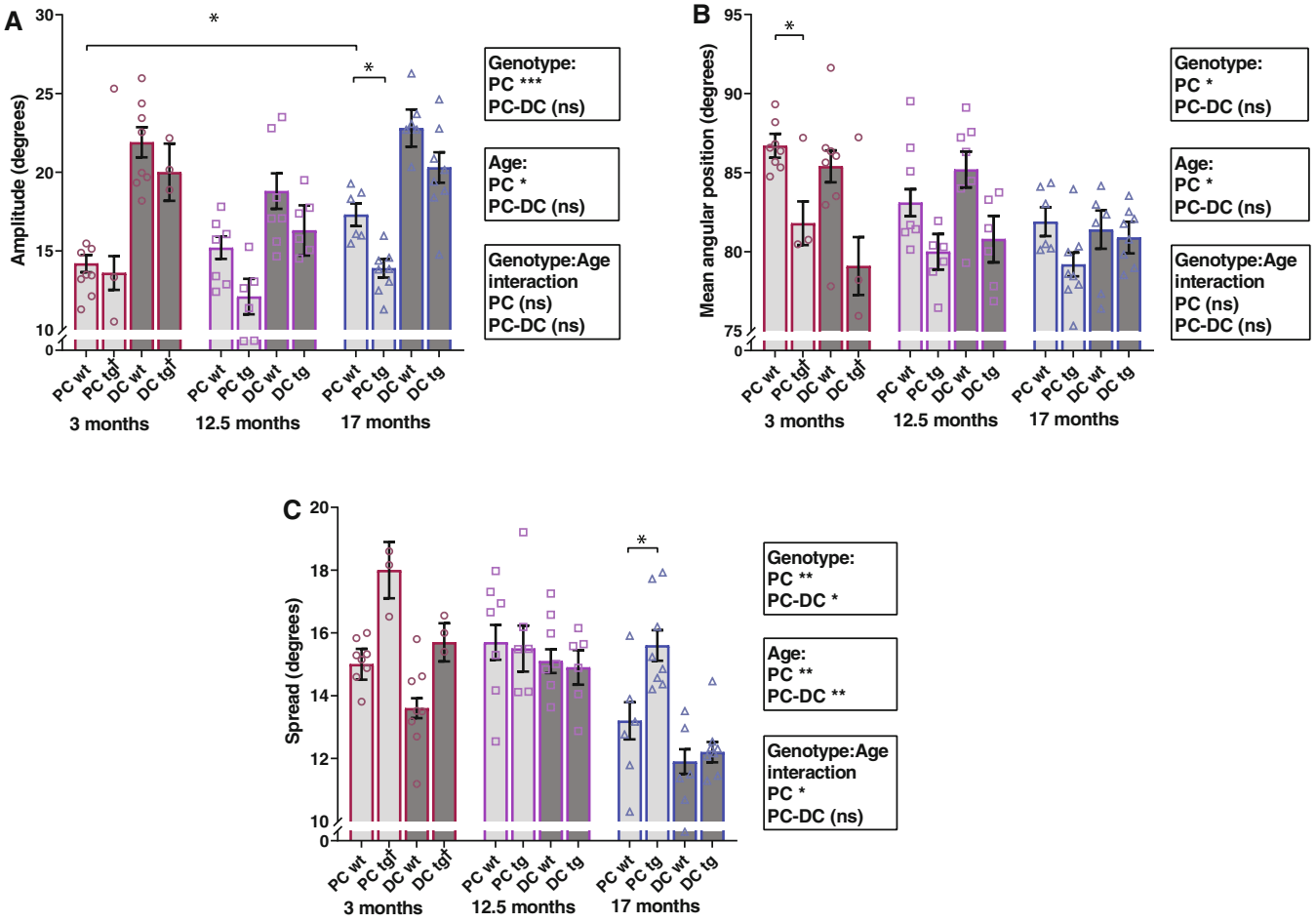

FIGURE 4 Mean angular position, amplitude and spread are affected by genotype and age. All significant differences are between 3xTg-AD and wildtype mice, unless otherwise specified. Panel A: Significant age and genotype effects were found in pre-contact mean angular whisker positions. Pairwise comparisons showed a significant difference in the 3-month age group. Panel B: Significant age and genotype effects were found in pre-contact whisker amplitudes. Pairwise comparisons showed a significant difference in the 17-month age group between 3xTg-AD and wildtype mice, as well as between 3 and 17-month wildtype mice. Panel C: Significant age and genotype effects were found in pre-contact whisker spread. Age and genotype effects were found in contact-related (PC-DC) spread. Pairwise comparisons showed a significant difference in the 17-month age group in (PC-DC) spread as well as between 12.5 and 17-month wildtype mice. The bars indicate the mean values from all the clips (degrees of freedom calculated from a linear mixed-effect model), with standard error bars. Asterisks mark significant values where $p \leq 0.05 = *$, $p \leq 0.01 = **$, $p \leq 0.001 = ***$. Data points show mean values for individual mice, indicated by circles for 3-month mice, squares for 12.5-month mice, triangles for 17-month mice. DC, during contact; PC, pre-contact; PC-DC, contact related behaviours. † indicates $n = 3$ mice.

genotype (F [1, 29.79] = 4.60, p = 0.040) and age (F [2, 28.04] = 6.79, p = 0.004) as (PC-DC) whisker spread was significantly higher in the 3xTg-AD mice than the wildtype mice at 17 months of age (p = 0.041; Figures 4C and 5). There was also a significant difference between 12.5-month and 17-month transgenic mice, with the 17-month transgenic mice reducing their spread more upon contact (p = 0.007) than the 12.5-month mice. Detailed statistical information for every comparison in PC-DC quantitative analyses can be found in Supplementary Table 4.

4 | DISCUSSION

As we hypothesised, the 3xTg-AD mice differed from age-matched wildtype mice in their whisker movements, both prior to and during object exploration. Specifically, we observed significant genotype differences in pre-contact whisking scores, mean angular position and whisking amplitude, as well as during-contact whisker spread, spread reduction scores and contact-induced asymmetry scores. We suggest that these observations may correspond to a whisker motor phenotype in 3xTg-AD mice from 3 months of age and a sensory or attentional deficit, associated with contact-related whisker movements, at 12.5 and 17 months of age.

4.1 | Pre-contact movements

Prior to any object contact, the whisking movements of the 3xTg-AD mice differed from the wildtype mice. The qualitative whisking scores showed that 12.5 and 17-month 3xTg-AD mice did not always make

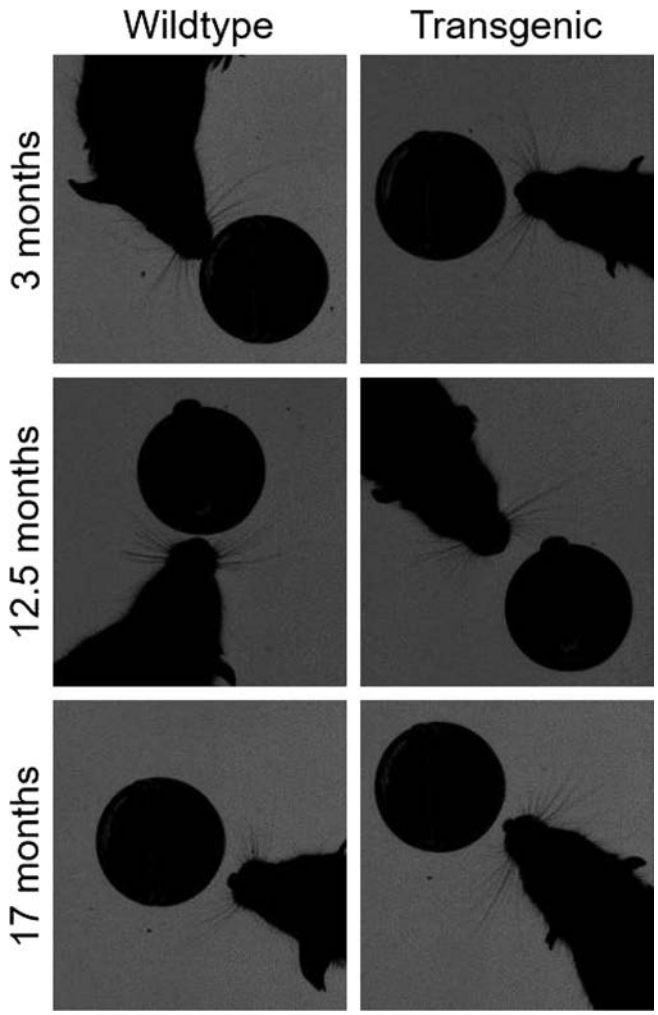

FIGURE 5 Whiskers are more spread out in 3xTg-AD mice during object contact. Video stills of representative mice are shown contacting the object, where whiskers are at maximum protraction. Whiskers of the wildtype mouse are positioned more forward towards the object and less spread out, compared with the 3xTg-AD mouse, especially at 3 and 17 months.

full retraction movements during whisking compared with the wildtypes (Figure 3A). Whisker tracking showed that mean angular positions of 3xTg-AD mice were consistently lower than the wildtype mice, and significantly so at 3 months (Figure 4B). Moreover, pre-contact amplitude was significantly lower in 17-month-old 3xTg-AD mice compared with the wildtypes. These findings suggest the presence of a motor phenotype in 3xTg-AD mice, from perhaps as early as 3 months of age. However, the exact age of this phenotype is unclear from our data and is likely to depend on the exact measure, since it varies between our measures of whisking, whisker angle and amplitude.

The 3xTg-AD mice are known for complex age-related motor abnormalities. The 3xTg-AD mice often perform better than non-transgenic mice in rotarod tasks (Blanchard et al.[42] at 6–7 months; Filali et al.[7] at 12–14 months; Chen et al.[43] at 6 months; Stover et al.[12] at 6 months; Garvock-de Montbrun et al.[13] at 16 months) and have longer stride lengths during locomotion.[12] However, other studies have shown that the stride length,[7,44] walking speed[12,44] and rotarod performance[44,45] can also be unaffected in 3xTg-AD mice. Indeed, locomotion speed was not significantly affected in our mice. However, it is worth noticing that we only measured locomotion speed in several frames as the mouse approached an object, therefore, it is not comparable to the gait analysis or rotarod and balance beam set-ups used by other studies. Some studies have even shown a reduced motor phenotype in 3xTg-AD mice. For instance, Garvock-de Montbrun et al.[13] showed that, despite the enhanced rotarod performance, 3xTg-AD mice at 16 months of age display a reduction in walking distance and speed compared with the wildtype mice in a balance beam task, suggesting an age-related decline in motor performance. Orta-Salazar et al.[46] also found a reduction in locomotion distance and time in 11-month 3xTg-AD mice in an open field test. Overall, we did not observe any evidence of an enhanced motor phenotype in the 3xTg-AD mice. In fact, our results are more in favour of a reduced motor phenotype, starting from reduced whisker angles at 3 months, and then seeing changes in whisking capacity at 12.5 and 17 months, later also showing up as reduced whisker amplitude at 17 months. One issue in the analysis of motor phenotypes in the 3xTg-AD mice is the background strain used. Background strains can have a significant effect on behavioural phenotypes[47] and the 3xTg-AD mice are available from the JAX Labs on three different backgrounds: B6;129 (Stock No. 004807), 129S4 (Stock No. 0319881), and C57BL/6J (Stock No. 033930). Recent research[48] suggests that the motor phenotype of the 3xTg-AD mice on the C57Bl6 background differs from that of the mice on the B6129 background that we used.

We observed variation in whisker movements between wildtype mice of different ages. Specifically, pre-contact whisker amplitude was significantly higher in 17-month wildtype mice compared with 3-month wildtype mice, and pre-contact mean angular position was significantly higher in 3-month wildtype mice compared with older mice. Very young mice (10-13-days-old) also have smaller whisker amplitudes than weaned (21-days-old) mice.[34] Therefore, there might be a tendency for pre-contact whisker amplitude to increase with age in wildtype mice. Although studies of age-related changes in whisker movements are few, Garland et al.[23] show a visible amplitude increase in older wildtype mice when testing Q175, Hdh Q150 and Hdh Q250 mouse models of Parkinson's disease (all mice tested at 10, 20 and 90 weeks, Hdh Q150 and Hdh Q250 mice also tested at 55 weeks; amplitude increasing at every age). They also show decreasing mean angular position in wildtype mice when testing the R6/2 CAG250 mice (decreasing from 8 to 10 weeks and from 12 to 18 weeks). However, these age-related changes were not statistically evaluated in their work. Investigating the changes in whisker movements over an animal's lifecycle would be a useful addition to this work.

Data from 17-month-old mice analysed by Simanaviciute et al.[11] were in agreement with our data, as they found that 17-month-old female 3xTg-AD mice had lower whisker angular positions than wildtype mice. However, they also found that retraction speed was significantly lower in the 3xTg-AD mice. While retraction speed was

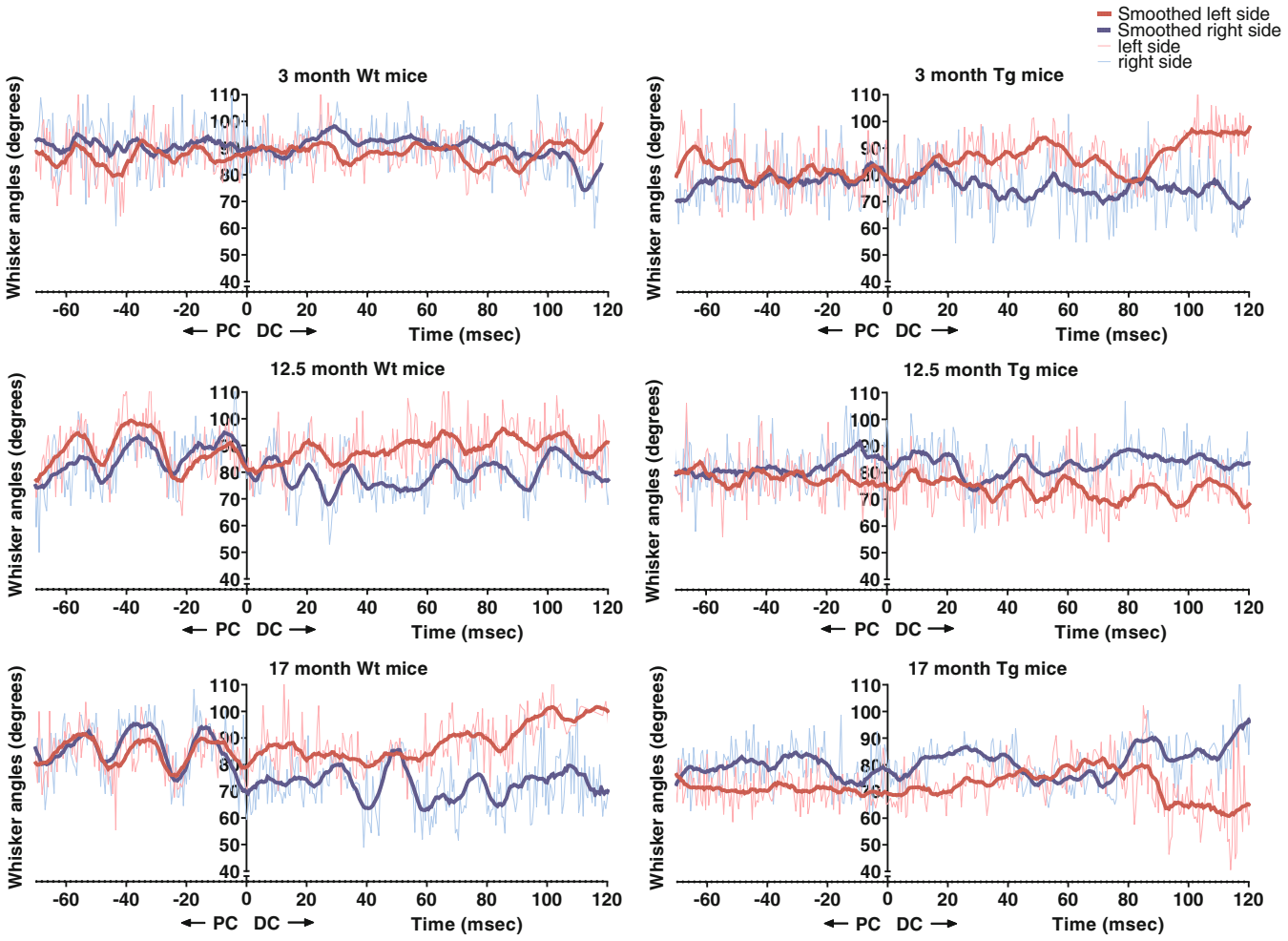

FIGURE 6 Example whisker angle traces of wildtype and 3xTg-AD mice at each age. Raw data points are shown in fine lines, and smoothed data (2nd order, 15 neighbours) are presented in thicker lines. Red colour traces are from the whiskers on the left side, and blue from the right side. 0 msec is the point of contact on the x-axis; therefore, left from the *Y*-axis is PC and right from the *Y*-axis is DC.

consistently lower in our 3xTg-AD mice compared with the wildtype mice (Supplementary Figure 1C), this difference was not significant in our analyses. Simanaviciute et al.[11] used per-clip measures for statistical analyses, whereas we use a stricter linear mixed effect model here. In statistical analyses, treating every trial as an independent data point can lead to pseudorepetition[49] and inflate the power of the statistical test. Therefore, per-trial, or, in this case, per-clip measures should not be used as independent data points, despite this often occurring in animal studies, especially where the sample size drops due to unforeseen experimental circumstances or data quality issues. In this case, we recommend a mixed-effect model that automatically determines degrees of freedom for the dataset instead of using standard parametric and non-parametric tests.[50,51]

4.2 | Contact-related movements

The 3xTg-AD and wildtype mice at all ages made robust object contact-related whisker movements, as indicated by a decrease in whisker speeds, spread, and increased amplitude and asymmetry following whisker contact (Figure 4B and C for amplitude and spread; Supplementary Figure 1 for all other parameters). Contact-related spread was affected in the 3xTg-AD mice, compared with the wildtype control mice. In the qualitative scoring, 12.5-month 3xTg-AD mice reduced whisker spread following contact less often than the controls. In the quantitative tracking, contact-related whisker spread was significantly higher in the 3xTg-AD mice than wildtypes at 17 months. 17-month 3xTg-AD mice also reduced their whisker spread following contact more than 12.5-month 3xTg-AD mice. It is unknown exactly what the sensory implications are of reducing whisker spread following contact, although it seems to play a role in increasing the number of whiskers contacting an object.[30] Why there is a difference in age in the spread reduction upon contact is not clear and demonstrates the need for more research in this field.

While some of these contact-related changes tend to be robust across animals,[11] some are still relatively variable and do not occur on every object contact. For example, 3-month-old wildtype mice show CIA significantly more often than other wildtype or 3xTg-AD mice

(Figure 3D), and HTA seems to be quite variable (Figure 3B). The reason for this is unknown, although it is likely due to variation in behaviour and motivation between individuals. Spread reduction, HTA and CIA have all been associated with orienting of the whiskers towards a region in space or an object, and hence with the animal's attention.[33] Contact-related whisker movement deficits observed in whisker spread and spread reduction could, therefore, imply an attentional deficit in 3xTg-AD mice. Attentional deficits have previously been documented in these mice in a visual task,[52] although in any sensory task it is challenging to separate attentional and sensory deficits.[52] Overall, our results suggest that contact-related sensory or attentional whisker movement deficits are likely to be present in 12.5 and 17-month-old 3xTg-AD mice.

We further compare our findings with those of other studies involving behavioural and cognitive tasks in Supplementary Table 5. Overall, in our study, and those of Stevens and Brown[14] and Fertan et al.[5,16,20] there is an early behavioural phenotype at 2–4 months old. This age group shows the most deficits in working memory and spatial learning,[5,14,16,20] despite being at the early stage of Alzheimer's disease. Our results show contact-related whisker movement differences at this age too – especially in contact-induced asymmetry scores and asymmetry, which may be associated with attentional or cognitive disturbances. We also describe an early motor phenotype, with pre-contact whisking amplitude significantly affected in these young mice. The 6-month group, which we did not test here, did not show any differences in the previous studies[14,16]; however, they observed some significant differences in working memory and spatial learning in the 12–13-month-old mice (Supplementary Table 5). We also observed differences in contact-related spread reduction and in pre-contact whisking scores at this age, perhaps indicating both motor and cognitive deficits. Surprisingly, deficits observed in 12–13-month-old mice were not maintained in older animals at 15 months in the studies by Stevens and Brown.[14] Indeed, previous studies do not show differences at later stages. We did not test 15-month-old animals; however, at 17 months, mice showed differences in both contact related and pre-contact measures, with whisking scores being maintained from the 12-13-month-old group. This suggests that in later stages of the disease, whisker movement measurements might be a better test to adopt than other, more standard behavioural tasks.

4.3 | Limitations

After the data selection process using our validated criteria, only three mice could be included in the 3-month 3xTg-AD group. We recognise that this sample size is low; however, we have kept the data with additional indication for a low sample size in figures and figure captions. We are also confident that the statistical method selected is appropriate to make the most of the uneven sample sizes. The difficulty of including more clips from this group might indicate that the 3-month 3xTg-AD mice behave differently from the other groups, since their clips did not often fit the selection criteria, while we were able to include a lot more clips from their control group. This could mean that we need to refine the data collection method to focus on collecting more clips from the young mice, given that in the previous studies from this laboratory as well as this study, the young female mice seem to be affected the most.

4.4 | Future recommendations

Following recommendations from Fertan et al.,[16] we observed the mice at different time points to examine age-related behavioural changes. However, since behavioural measures can be relatively variable, observing the same mice at each time point in a longitudinal study might be more beneficial than observing different groups of mice in a cross-sectional study. Nevertheless, it is rather difficult to conduct such a study, especially to 17 months, due to the increased mortality rates in older 3xTg-AD mice.[19] In addition, repeat testing of the same animal can impact behavioural tasks, as animals will habituate and learn tasks over time, which may affect their behaviour.[53] Indeed, we have previously shown that a mouse model of anxiety has different whisker movements to control mice[24]; therefore, an altered sensitivity to stress is likely to affect our results. The lack of automation of our set-up may also confound testing over different ages, while here we made sure that all data was collected over a period of just a few days, with all the equipment kept the same throughout.

As there are clear sex differences in 3xTg-AD mice[16,18] and whisker movements differ between sexes in other mouse models,[11,23,25] investigating whisker movements in male 3xTg-AD mice at different ages would be beneficial. It would also be interesting to investigate whether the amyloid quantity in the barrel cortex is related to whisking impairment in 3xTg-AD mice. We have previously shown that models of cortical development disorders have whisker movement deficits (in Robo3R3–5-CKO and RIM-DKOSert models), suggesting that cortical differences can affect whisker movements. However, our previous studies have also shown differences in whisker movements in non-neurodegenerative mouse models (MCAO model of stroke and heterozygous Reeler mice, Simanaviciute et al.[11]), which would suggest that whisking impairment is not specifically related to neurodegeneration and amyloid levels in the cortex, but likely caused by many changes in the brain.

In agreement with Simanaviciute et al.,[11] measuring whisker movements is a quick, robust and semi-automated way to capture motor, sensory and cognitive behaviours in rodents. While the qualitative scoring of whisking, spread reduction, CIA and HTA were valuable at assessing whisker behaviour, they require manual scoring and are relatively time-consuming to complete. We wanted to assess whether measuring spread automatically was a more sensitive method than manual scoring, and it has shown differences at more advanced disease stages than were found by manual scoring. Therefore, it might be worth developing ARTv2 to measure these qualitative scorings automatically. Developing quantitative data and better analytical methods will improve the robustness of repeated testing. Our findings differed from Simanaviciute et al.,[11] probably due to the difference in

statistical methods. We suggest using a linear mixed effect model for future analyses (package lme4 in R-studio, Bates et al.[37]) as we did here, which makes the most of smaller and uneven sample numbers, without assuming per-clip or per-trial independence. Small improvements in automation and analysis techniques will also help to develop whisker movements as a powerful behavioural measurement tool, with particular benefits in capturing behavioural deficits in mouse models that show complex or subtle phenotypes, such as in the 3xTg-AD mouse model. Indeed, the barrel cortex has been found to contain amyloid plaques in several mouse models of AD, including Tg19959 mice at 3 months,[54] APP transgenic mice Tg2576 at 17.5 months[55] and APP/PS1 mice at 19.5–21 months of age.[56] In order to understand the relationship between amyloid levels and whisker movement impairments, it would be beneficial to study whisking in these mouse models.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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SUPPORTING INFORMATION

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Maternal immune activation affects female offspring whisker movements during object exploration in a rat model of neurodevelopmental disorders

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ABSTRACT

Poly I:C rat offspring are used to investigate the effects of *in utero* exposure to maternal immune activation (MIA) and have been suggested as a model of neurodevelopmental disorders (NDD). The behavioural symptoms of this model are diverse and can vary with external factors, including the choice of background strain and husbandry practices. Measuring whisker movements provides quantitative, robust measurements of sensory, motor and cognitive behaviours in rodents. In this study, whisker movements were investigated in 50-day-old male and female offspring of MIA-exposed rat dams and compared to age-matched offspring of control (vehicle) dams. Rat offspring were filmed using high-speed videography in a sequential object exploration task with smooth and textured objects. Poly I:C treatment effects were found in female offspring that did not increase whisker mean angular position during object exploration, especially for the smooth object, indicating an attentional deficit. Whisker tracking during object exploration is demonstrated here, for the first time, as a useful, quick and non-invasive tool to identify both treatment effects and sex differences in a model of MIA-induced NDDs.

1. Introduction

Neurodevelopmental disorders (NDDs), including schizophrenia, attention deficit hyperactivity disorder and autism spectrum disorder, affect the development of the nervous system and normal brain function. This can have wide-ranging consequences, impacting cognition, emotion, learning, self-control and memory. In pre-clinical studies, maternal immune activation (MIA) rodent models of NDDs have been found to reflect the natural pathogenesis of the NDDs and their symptoms (Woods et al., 2021). A widely used method to induce MIA is gestational exposure to the viral mimetic and Toll-like receptor 3 agonist polyinosinic:polycytidylic acid (Poly I:C, Bucknor et al., 2022). Offspring of Poly I:C dams express behavioural deficits in sensorimotor gating, selective attention, social behaviour, exploratory behaviour, working memory, and cognitive flexibility (Meyer, 2014; Potter et al., 2023). However, the Poly I:C rodent model is challenging, as behavioural findings can vary depending on the rodent genetic background, source and dose of Poly I:C, as well as the gestational timing of treatment (Mueller et al., 2018; Kowash et al., 2019; Murray et al., 2019). Even the type of caging system can affect maternal behaviour and the behaviour of adult offspring (Mueller et al., 2018). Consequently, it is imperative to select a robust and highly quantitative behavioural test to identify such complex behavioural phenotypes.

We posit that measuring whisker movements could be such a robust test. Whiskers are an established sensorimotor model in neuroscience (see Adibi, 2019 for a recent review), and the precise measurement of whisker movements can be captured quickly without any animal training (Simanaviciute et al., 2022), unlike in many other behavioural tasks. Furthermore, measurements are highly granular, including angles and speed. This is in contrast to counts and durations which are more common in behavioural testing, such as the tasks recommended for measuring attention by Lustig et al. (2013). Measuring rodent whisker movements has previously revealed motor, sensory and cognitive deficits in mouse models of neurodegenerative disease (Grant; Garland et al., 2018; Simanaviciute et al., 2020, 2022), although it is probably not possible to disentangle these factors using the current set up. This

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method has also identified behavioural phenotypes earlier than any other behavioural test, e.g., in a R62 Huntington's Disease mouse model (Garland et al., 2018). Measuring whisker movements during object exploration can identify sensory and attentional deficits (5xFAD mice in Grant et al., 2018; 3xTg-AD mice in Simanaviciute et al., 2022), and reveal sex differences in mouse models of Alzheimer's (Grant et al., 2018) and Huntington's disease (Simanaviciute et al., 2020), which makes it especially aligned for the study of NDDs. Indeed, Lins et al. (2019) specifically recommend investigating sex differences in MIA research, since rat models of NDDs exhibit sex-dependent phenotypes (Snigda et al., 2011; Leger and Neill, 2016; Nikolić et al., 2017; Casquero-Veiga et al., 2023; Potter et al., 2023).

Here, we applied our established mouse whisker measurement protocol (Simanaviciute et al., 2020, 2022) to the rat Poly I:C model to investigate the effects of *in utero* exposure to MIA on whisker movements in offspring at postnatal day (P) 50. We investigated whisker movements before and during object exploration, and examined differences between treatment, sex and object texture. We predict that measures of whisker movements would be sensitive to both treatment and sex effects.

2. Materials and methods

2.1. Animals

Details of the rat MIA model, including information about animal treatment and housing were reported following the guidelines from Kentner et al. (2019) and can be found in Potter et al. (2023). For our study, 11 dams were pseudo-randomised to treatment group (Excel v2004 random number generator, Microsoft, USA). Dam sample sizes were calculated using the statistical package G*Power v3.1.9.2. Whisker movements of the offspring of these dams were measured once they reached early adulthood, at ~ P50 (47–53) (Supplementary material A, Table S1) when whisker movements are adult-like (Grant). From a total of 24 MIA offspring rats, 11 were female and 13 male, and from 26 control rats, 14 were female and 12 male. Half of the rats were cross fostered as part of a satellite study; however, this had no significant effect on whisker metrics (Supplementary material B, Tables S2 and S3) nor on other adult behaviours (Potter, 2021). Therefore, data were combined and cross-fostering was not investigated further.

2.2. Experimental procedures

All experimental procedures were carried out at the University of Manchester Biological Services Facility, in the light phase of the daily cycle (standard 12h light:dark cycle, lights on at 7:00am). Experiments were performed under Home Office UK project licence (number P473EC3B1) in accordance with the Animals (Scientific Procedures) Act UK 1986. Before being involved in this study, all animals had been exposed to other tasks at P35 (~15 days before this study, as described in Potter et al., 2023), including novel object recognition (NOR), elevated plus maze and social interaction tasks.

For high-speed filming of whisker movements, a sequential object exploration task was adopted (Supplementary material C, Fig. S1), exact details of which can be found in Landreth et al. (2021). Observers were blind to the rat treatment group throughout the video collection and analysis process. Video clips were selected for analysis based on criteria developed by Grant et al. (2014); that the head was level, the whiskers in view and the rat be travelling toward the object in the pre-contact (PC) section of the clip and the whiskers contacting the object in the during-contact (DC) section of the clip. Clips were tracked using the Automated Rodent Tracker (ARTv2; Gillespie et al., 2019). 2–12 whiskers were detected in each frame (with 5–6 whiskers on each side being usual). 1–4 video clips per rat were included in data analyses (Supplementary material A, Table S1), giving a total of 114 clips, including both PC and DC sections. PC sections ranged from 100 to 291 frames per clip and DC sections ranged from 100 to 459 frames. Whisker metrics included both PC and PC-DC measures of mean angular position (mean whisker angles), whisker amplitude (standard deviation of angular positions multiplied by $2x\sqrt{2}$), whisker asymmetry (difference between left and right angular position), whisker spread (mean standard deviation of all tracked whiskers), and retraction and protraction speeds (mean speed of whiskers moving backwards and forwards, respectively). Full definitions for these metrics can be found in Simanaviciute et al. (2020).

2.3. Statistical analyses

Measures of the PC whisker variables were analysed first. Changes in whisker movements during object exploration were analysed by subtracting the DC measures from the PC measures (PC-DC), as per Simanaviciute et al. (2022). Examining PC-DC metrics reveals common contact-related whisker behaviours (Fig. 1), including increasing the number of whisker contacts (increasing protraction angles and decreasing whisker spread during contact), while ensuring light whisker contacts (increasing asymmetry during contact) over a longer period of time (by reducing whisker speeds during contact) (see Grant and Goss, 2022 for a full review).

Linear Mixed-Effects Models (lme4 in R Studio version 1.1.456) were used to analyse the effect of Poly I:C treatment, sex and object texture (or order) on all PC and (PC-DC) whisker variables. The degrees of freedom were approximated using a Kenward-Rodger's method and could fall anywhere between the number of clips (n = 114) and number of individuals (n = 50). A significance value of $p < 0.05$ was used throughout and adjusted in pairwise comparisons with Tukey's method. Data and code used for analysis is referenced in Supplementary material E.

3. Results

In analyses with males and females combined, both MIA-treated and control rat offspring exhibited the common contact-related behaviours that we have previously observed in mice. During contact with an object, whisker retraction speed, protraction speed and spread were consistently reduced (Supplementary material D, Supplementary Figs. S2C, D, and E), while amplitude and asymmetry increased (Supplementary material D, Supplementary Figs. S2A and B). We next tested for Poly I:C treatment and sex effects. There was no effect of treatment, but a sex effect in PC mean angular position was detected (Fig. 2A, Supplementary material D, Table S4), with female rats having higher PC mean angular positions than males (treatment: $F_{1,\ 41.224} = 2.446$, $p = 0.125$ sex: $F_{1,\ 41.224} = 5.240$, $p = 0.027$; interaction: $F_{1,\ 41.224} = 1.034$, $p = 0.315$). There were no further significant effects of treatment or sex in any of the other whisker variables (all p-values >0.05; Fig. 2, Table S5).

3.1. Female offspring

Since there was a sex effect, treatment and object texture (or order) was then investigated in males and females separately. (PC-DC) mean angular position in females had a treatment and object texture effect (treatment: $F_{1,\ 20.750} = 5.454$, $p = 0.030$; texture: $F_{1,\ 51.992} = 4.573$, $p = 0.037$; interaction: $F_{1,\ 51.992} = 4.338$, $p = 0.042$, Fig. 2B, Supplementary materials D, Table S6). Post-hoc comparisons showed that female MIA offspring rats contacting the smooth (first) object had significantly higher (PC-DC) mean angular positions compared to female control rats contacting the smooth object ($p = 0.049$) and MIA offspring rats contacting the textured (second) object ($p = 0.034$). This indicates that female MIA offspring rats show the opposite of the predicted contact-related changes in their whisker angular position on the smooth object. This effect can be visualised in the example whisker traces in Fig. 3 (corresponding video clip in Supplementary Video Clip), showing that whiskers of female MIA offspring rats were less protracted during object contact, especially when contacting the smooth (first) object (Fig. 3B).

While there was no treatment effect, a texture (or order) effect was

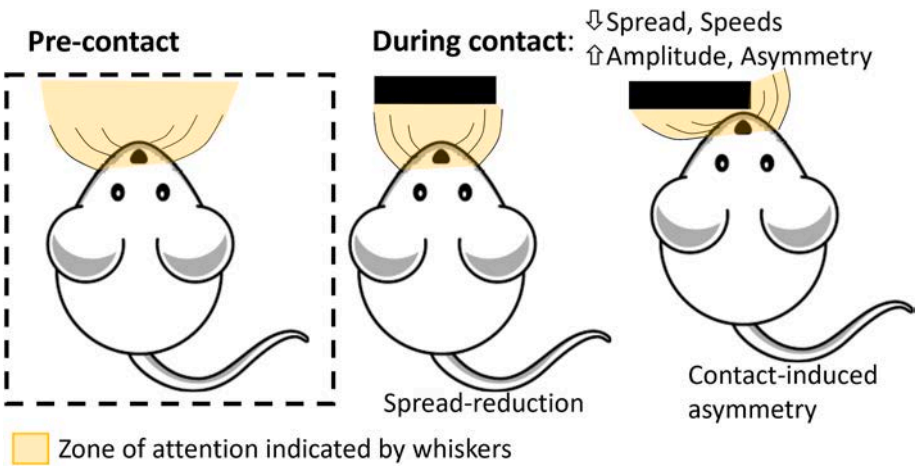

Fig. 1. Summary whisker positional changes observed pre-contact and during contact. Object contact causes increases in whisker amplitude and asymmetry (termed contact-induced asymmetry) and a reduction in whisker spread and movement speeds (both retraction and protraction speed). The area covered by the whiskers can be thought of as a zone of attention.

found in (PC-DC) asymmetry in female rats (treatment: $F_{1,\ 20.750} = 0.086$, $p = 0.772$; texture: $F_{1,\ 51.992} = 4.274$, $p = 0.044$; interaction: $F_{1,\ 51.992} = 0.061$, $p = 0.806$, Fig. 2C–Supplementary material D, Table S6). While all other female rats increased their whisker asymmetry during object contact, female MIA offspring rats contacted the smooth object more symmetrically, indicated by positive (PC-DC) values in Fig. 2C and a smaller separation between red and blue traces in Fig. 3B (see also Supplementary Video Clip). There were no further significant effect of treatment nor object texture on any PC whisker metrics, nor other PC-DC metrics (all p-values >0.05).

3.2. Male offspring

There was no treatment effect in male rat (PC-DC) mean angular position, but object texture (or order) was significantly different (treatment: $F_{1,\ 19.980} = 0.307$, $p = 0.586$; texture: $F_{1,\ 53.768} = 6.856$, $p = 0.012$; interaction: $F_{1,\ 53.768} = 1.517$, $p = 0.223$, Fig. 2B–Supplementary material D, Table S6). While all other male rats pushed their whiskers more forward during object contact, male control rats on the smooth object reduced their mean angular positions (Fig. 2B). There were no further treatment or object texture (order) effects in male offspring rats in any PC and or (PC-DC) whisker measures (all p-values >0.05).

4. Discussion

We investigate here, for the first time, the effect of *in utero* exposure to MIA on whisker movements in P50 offspring, a rat Poly I:C model. When male and female rats were grouped, we saw no effect of Poly I:C treatment on any of the PC or (PC-DC) whisker metrics (Supplementary material D, Supplementary Fig. S2). In agreement, Potter et al. (2023) also found no effect of Poly I:C treatment in adolescent or adult male or female MIA rats in a NOR task used to assess visual learning, and an elevated plus maze task used for measuring anxiety-related behaviour.

However, when we split our data by sex, we found significant treatment effects in female offspring. Sex differences are important to consider in any research and is especially relevant when modelling NDDs, which are expressed differently in males and females (Kokras and Dalla, 2014). We agree with the importance of including both male and female animals in studies and evaluating their neurological symptoms separately, as treatment effects can be masked by innate sex differences. Given the young age of our animals (~P50), some sex-differences may be due to different developmental trajectories, and we would recommend repeating this study at more ages in order to fully describe their sex differences.

We observed that female MIA offspring rats did not increase their mean angular position during contact with the smooth (first) object, implying they did not engage in the contact-related behaviours that we would usually expect. Conversely, female MIA offspring rats contacting the textured object and control rats touching the smooth object all increased their mean angular position during contact. Both MIA and control female offspring rats contacting the smooth object engaged in contact-induced asymmetry less than male rats (Fig. 2). Positioning whiskers more forward during object contact is associated with focussing of attention (Arkley et al., 2014; Mitchinson et al., 2007, Fig. 1). We would typically expect to see increases in both forward whisker mean angular positions and asymmetry during contact (Berg and Kleinfeld 2003; Grant et al., 2009; Mitchinson et al., 2007). Since these behaviours were absent in the MIA female offspring rats contacting the smooth object, it may suggest abnormal behaviours and attentional deficits in MIA female offspring. We have observed the same behaviour in female 5XFAD mice, a model of Alzheimer's Disease, which was also not present in males (Grant et al., 2020). Potter et al. (2023) also found multiple deficits in attention and problem solving in female MIA offspring rats in more classic behavioural tasks, which further supports our conclusion that attention and executive function are likely to be impacted by the Poly I:C treatment in female rats.

This is the first time we have observed significant treatment effects on whisker movements with different objects. An identical sequential object exploration task was used by Landreth et al. (2021) in a sub-chronic PCP rat model of schizophrenia and did not reveal any differences between the smooth and textured objects. It is worth noting that the objects were very similar and only differed in colour and texture (Supplementary material C, Fig. S1), thus, the novelty of the second object is not especially pronounced. However, the number, type, texture and novelty of objects may affect rodent behaviour in object exploration tasks and should be considered when looking for MIA treatment effects. In our animals, we suggest that treatment and sex differences may primarily manifest during whisker exploration of the first, novel object presented to them. This means that whisker movements could be measured with only one object in the future, further simplifying the set-up for this method. Indeed, we show here that measuring whisker movements offers a quick, non-invasive, quantitative tool that is sensitive enough to identify treatment and sex effects during object exploration in an offspring model of NDDs caused by MIA.

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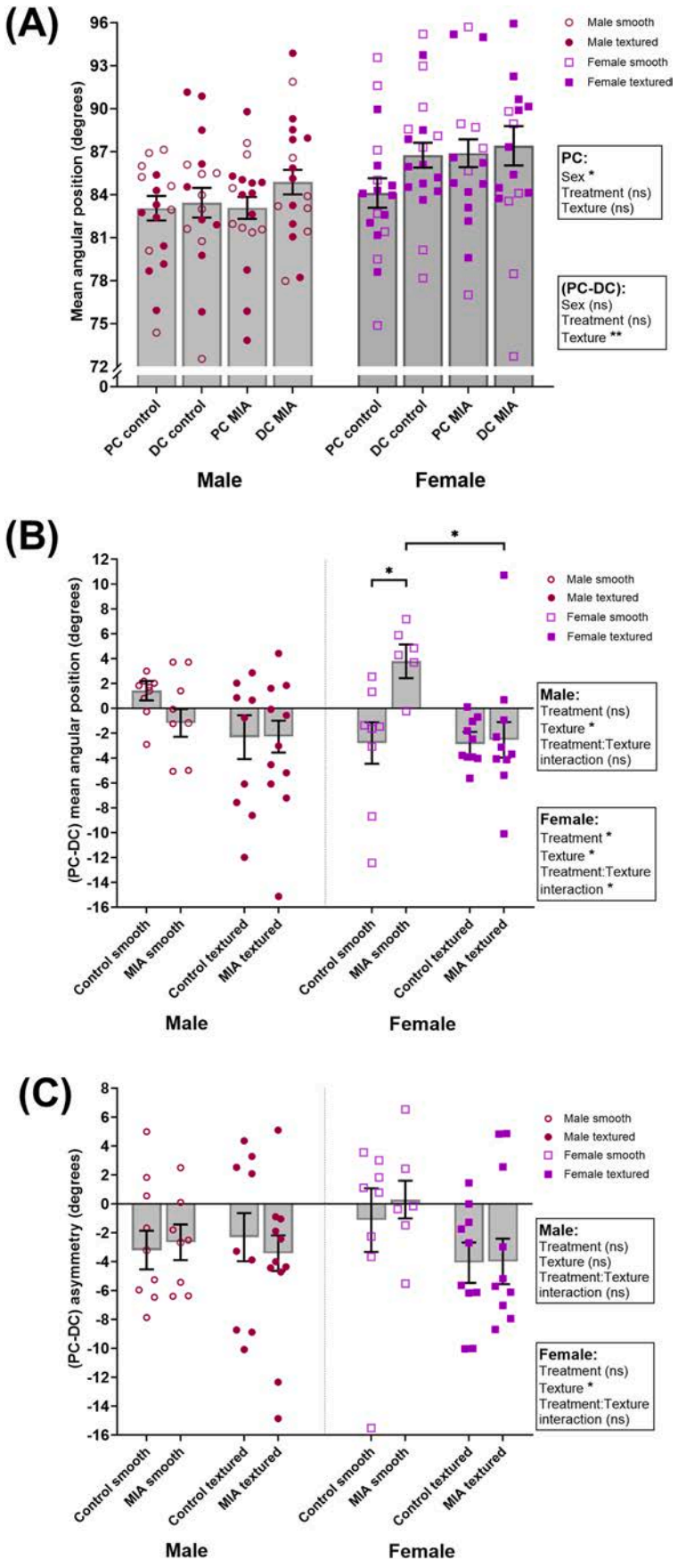

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Fig. 2. MIA offspring rat mean angular position (A and B) and asymmetry (C) are affected by treatment and object texture. A) Significant sex effects were found in PC whisker mean angular position and object texture effects were found in (PC-DC) mean angular position. B) In males, there was a main effect of object texture in (PC-DC) mean angular position. In females, there were effects of treatment, object texture and their interaction in (PC-DC) mean angular position, while pairwise comparisons showed that MIA offspring rat smooth texture group was significantly different to control rats exploring the smooth object and MIA offspring rats exploring the textured object. C) There was a significant effect of object texture in (PC-DC) asymmetry in females, independent of treatment. Males and females were analysed together in A and separately in B and C. The bars indicate the mean values from all the clips (degrees of freedom calculated from a linear mixed-effect model), with error bars representing SEM. Data points show mean values for individual rats, indicated by open circles for male rats investigating smooth object, filled circles for male rats investigating textured object, open squares for female rats investigating smooth object, and filled squares for female rats investigating textured object. PC = pre-contact, DC = during contact, (PC-DC) = contact related changes. Asterisks mark significant values where $p \leq 0.05 = *$, $p \leq 0.01 = **$ and n.s. is not significant. Sample sizes: 13 MIA male and 11 MIA female offspring rats, 12 male control and 14 control female rats.

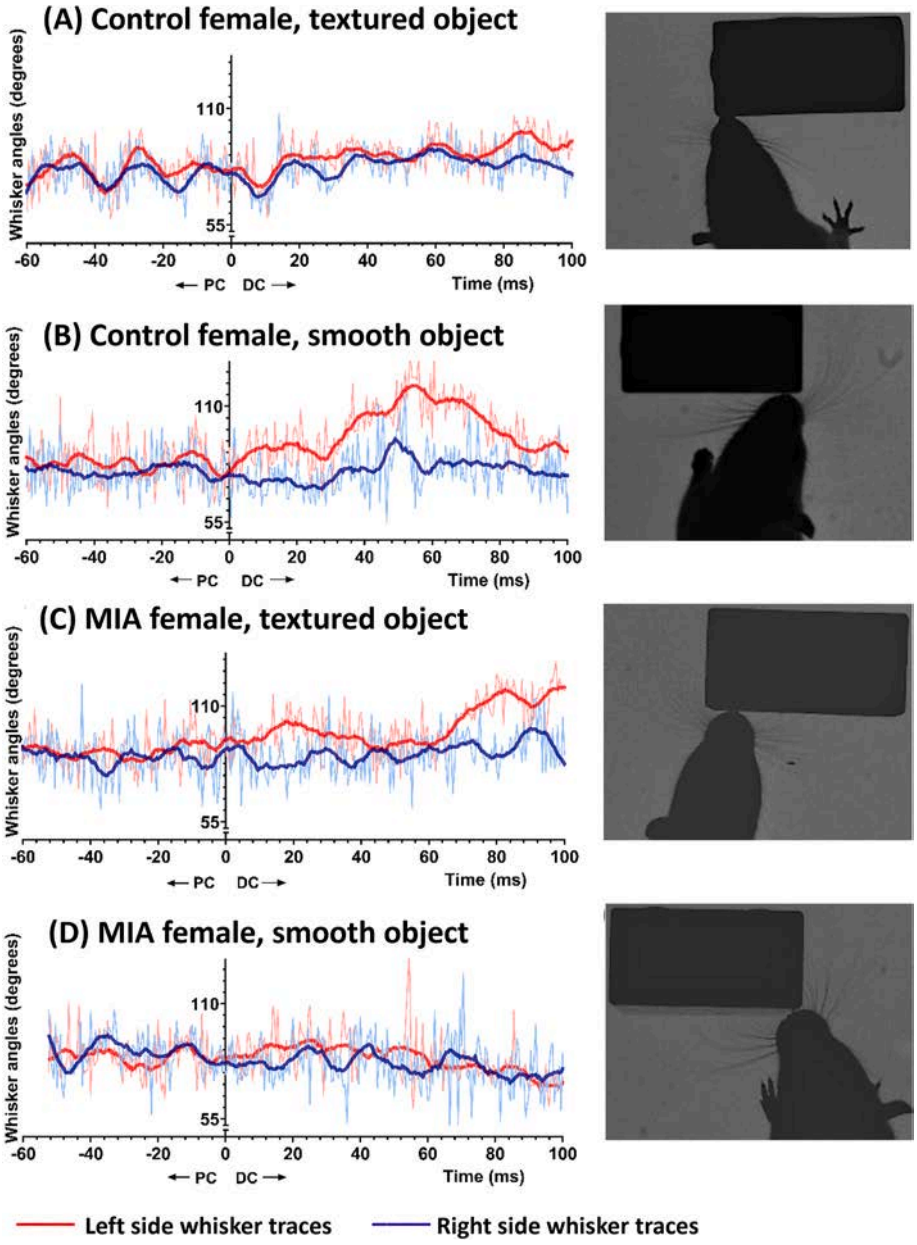

Fig. 3. Example whisker angle traces and video stills of female offspring exploring an object. Control females (A and C) show predicted object-related whisker behaviours, indicated by asymmetric positioning of the whiskers and high whisker angular positions (e.g., more forward-reaching whiskers) following an object contact. However, the female MIA offspring rat whiskers were more symmetric and less protracted during object contact, especially when contacting the smooth (first) object (B). Whisker traces are shown on the left hand panels. Raw data points are shown in fine lines, and smoothed data (2nd order, 15 neighbours) are presented in thicker lines. Red colour traces are from the whiskers on the left side, and blue from the right side. 0 ms is the point of contact on the x-axis; therefore, left from the Y-axis is pre-contact (PC) and right from the Y-axis is during-contact (DC). Example video clips (one per trace) used here can be found in Supplementary video. Video stills (right hand panel) are selected here when the whiskers are contacting the object at their maximum protraction. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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CRediT authorship contribution statement

Ugne Simanaviciute: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Harry G. Potter:** Data curation, Investigation, Methodology, Writing – review & editing. **Reinmar Hager:** Methodology, Project administration, Resources, Supervision, Writing – review & editing. **Jocelyn Glazier:** Methodology, Project administration, Resources, Supervision, Writing – review & editing. **Emma Hodson-Tole:** Supervision, Writing – review & editing. **John Gigg:** Conceptualization, Methodology, Project administration, Resources, Supervision, Writing – review & editing. **Robyn Grant:** Conceptualization, Methodology, Project administration, Resources, Software, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

We declare no conflicts of interest.

Data availability

Data is linked in Supplementary Material. Video clips are available upon request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2024.100807.

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