The effect of genotype on attention bias in rhesus macaques, *Macaca mulatta*, as a welfare indicator

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Abstract

Rhesus macaques, *Macaca mulatta*, are one of the most commonly used non-human primates in biomedical research in the UK. Their welfare is of great concern for both ethical and quality-of-science reasons. Attention bias (AB), a measure of cognitive bias, assesses whether an individual is stressed, predicts vulnerability to stress, and identifies the effectiveness of interventions to improve well-being. In both humans and macaques, genetic factors can result in variation of behavioural traits, attentional processes and susceptibility to stress-related neuropsychiatric disorders.

Here, sixty-five female macaques were genotyped for known variants in the following genes: serotonin transporter (*5-HTTLPR;* short and long allele), tryptophan hydroxylase 2 (*TPH2;* short and long allele), monoamine oxidase A (*MAOA;* 5-, 6- and 7-repeat allele) and mu-opioid receptor (*OPRM1;* C and G allele). Additionally, sequencing was utilised to identify novel SNPs in the dopamine receptor D4 (*DRD4*). The *5-HTTLPR* short-, *TPH2* long-, *MAOA* 7-repeat and *OPRM1* G-allele are low-expressing alleles leading to lower levels of circulating neurotransmitters in the brain, and have been shown to be linked to anxiety, coping mechanisms and vulnerability to stress in macaques. Twenty-nine of the genotyped macaques underwent AB testing with conspecific stimuli (aggressive *vs.* neutral facial expression, Experiment 1) and stimuli of the facilitie's veterinarian (photograph *vs.* pixelated photograph, Experiment 2). Additionally, to assess the effectiveness of habituation to change cognitive responses and improve well-being, females underwent different amounts of habituation to the veterinarian. Video footage was blind-coded for gaze towards stimuli and female's behaviour following AB tests was recorded. Associations between AB scores, other behavioural measures, and these genetic polymorphisms were investigated in the *R* package and in *SPSS*.

In Experiment 1, females were more avoidant of aggressive conspecific stimuli when they carried the *HTTLPR* short- or *MAOA* 5- and 6-repeat allele and in Experiment 2 females were more avoidant of vet photographs when they carried *HTTLPR* long- plus *TPH2* short alleles only. *DRD4* T-allele carriers showed increased amounts of aggressive behaviours and *MAOA* 7- repeat allele carriers showed increased amounts of affiliative and reduced amounts of maintenance behaviours. Genotype did not have an effect on habituation in Experiment 2, but the more habituation females received the less vigilant they became for the vet photograph.

This was the first study showing that genotype impacts on AB in macaques. Advice on further development of methods and future studies is given.

Contents

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Chapter 1. Introduction

Rhesus macaques and animal welfare in captivity

Out of the non-human primates, rhesus macaques, *Macaca mulatta*, are the most widely distributed and ecologically adaptable, presenting remarkable variability in social organisation and individual behavioural strategies (Charkraborty *et al.* 2010). Rhesus macaques form highly hierarchical and nepotistic societies (Thierry 2000) where males are dominant over females and females form matrilineal hierarchies; their daughters inherit the rank of the mother (Berman 1982). They share many genetic, physiological and behavioural traits with humans and other non-human primates and hence provide a perfect model for the comparative analysis of genetic and environmental factors underlying normative as well as pathological outcomes in development (Bennett *et al.* 2002). This makes them the most commonly used non-human primate in biomedical research, with the UK alone using approximately 1950 macaques annually (Home Office 2013). The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) stated that the use of primates in research is of particular concern due to their highly cognitive abilities as well as complex social, behavioural and psychological needs (NC3Rs 2011).

It is increasingly accepted that animals are capable of experiencing emotions and that the valence of their affective state is an important dimension of their welfare (Paul, Harding & Mendl 2005; Boissy *et al.* 2007; Mendl *et al.* 2009; Veissier *et al*. 2009). The valence of an emotional (or affective) state is whether it is a positive or negative emotion ('sad' or 'happy'; Watson *et al.* 1988; Russell 2003). Suomi (2006) stated that even slight changes of their physical or social environment can result in consistent responses of individuals by showing profound emotional, psychological or physiological distress. The factors underlying those individual differences in responses are not well understood but are extremely important in order to intervene and improve the well-being of those individuals.

Excessive emotional sensitivity in humans characterizes reactive aggression, which is triggered by negative emotions and life experiences, anger and anxiety (Robinson & Wilkowski 2010). This appears to be the result of an exaggerated threat perception as well as an inability to control the resulting emotional state (Blair *et al.* 2006). Additionally,

in humans, females are under increased risk for depression compared to males and macaques show a consistent effect, where females perform more self-directed behaviour (a measure of affective psychopathology or despair) following repeated stress (Spinelli *et al.* 2012). Rhesus macaques also display extreme differences in behaviour and biological responses to stress and it has been shown that genetic as well as environmental factors contribute towards those (Suomi 2006). Currently, psychological welfare of captive animals is hard to assess, usually relying on presence or absence of stereotypical behaviours (Mason & Rushen 2008). Stereotypic behaviour is defined as a repetitive, invariant behavioural pattern with no obvious goal or function and only occurs in captive animals (Mason 1991). However, stereotypical behaviour is not always indicative of bad welfare at the current point and varies between species and individuals (Mason & Latham 2004). For example, even if welfare is improved at the present point, performance of stereotypic behaviour is extremely hard to extinguish.

If increased anxiety and an inability to control emotional state leads to increased aggression, this is an important factor to consider for the welfare of any individual in captivity. This highlights the urgent need for new ways to measure and assess captive animal welfare in order to reliably identify psychological suffering at a much earlier point, giving caretakers a window of opportunity for rectification of conditions at an early stage. In order to be able to avoid the perceived inability to control emotional state, care-takers and those responsible for the captive animal have to be able to identify when an individual is at risk to perceive stimuli as a threat. Attention bias tests exactly that.

Attention bias

One recent opportunity to help improve ways of assessing welfare has been suggested to be by investigating cognitive components of affect (Doyle *et al.* 2011). The term 'cognitive' refers to information processing, attention, learning, memory and decision making (Shettleworth 1998). Cognitive processes are known to be influenced by an individual's cognitive state as well as the valence of an individual's emotional state (Mendl *et al.* 2009). An individual's emotional state influences attention, memory retrieval and judgment about future events or ambiguous stimuli (Mathews & Macleod 1994; Minkea *et al.* 1998; Eysneck *et al.* 1991; MacLeod & Byrne 1996; Mendl *et al.* 2009). Affective states are also thought to impact on pre-conscious attentional processes in humans, meaning that the initial response in attention to something is guided by

underlying affective state and how an individual 'feels' (Mathews & Macleod 1994; Bradley *et al.* 1995). Being in a negative emotional state puts individuals at increased risk to interpret new information in a negative or threatening way (Bar-Haim *et al.* 2007) and indeed, advances in research have shown that a range of animals judge ambiguous information differently according to the conditions they have previously been exposed to (Bateson & Matheson 2007; Burman *et al.* 2008; Matheson, Asher & Bateson 2008; Mendl *et al.* 2009; Doyle *et al.* 2010). Hence, subjective emotional influences can bias reward-processing and decision making, even if those emotions are irrelevant to the current decision (Lerner & Keltner 2000) and those biases towards and away from specific stimuli predict emotional vulnerability (Fox, Ridgewell & Ashwin 2009). Being vigilant for threat-related material is associated with emotional vulnerability, whilst a bias to avoid such stimuli is associated with resilience (Fox 1993). Humans for example, spent more time looking at angry faces compared to happy or neutral faces (Fox, Russo & Dutton 2002) and depressed individuals demonstrate increased elaboration of negative material as well as a tendency to interpret ambiguous material in a mood-congruent manner (Mathews & McLeod 2005). Anxiety is linked to a more negative judgement of ambiguous information as well as increased expectation of negative future events (Eysenck, Payne & Santos 2006; Richards *et al.* 2002).

Therefore, measures of attention, memory and judgment can indicate an individual's emotional state (Mendl *et al.* 2009) and we can infer an individual's inner state and the valence of affective state by judging their cognitive bias (Bethell *et al.* 2012a). Further, modifying cognitive biases should change an individual's reactivity to emotion-eliciting events and modulate their ability to regulate negative affect (Tran, Hertel & Joorman 2011). This means that cognitive bias can reveal emotional vulnerability of rhesus macaques and additionally, if habituation to an aversive stimulus could result in a modulating effect to regulate negative affect of this stimulus, this would potentially improve their welfare by identifying emotion eliciting stimuli and allowing them to regulate their reaction to it through habituation, where possible.

Looking time tasks involve an individual being presented with visual stimuli and the measurement of the way it corresponds towards those stimuli by eye gaze (Winters, Dubuc & Higham 2015). The pattern of eye gaze then leads to interpretation of the individual's perceptive or cognitive abilities (Spelke 1985) and the longer an individual

looks at a stimulus presented, the more interesting it is thought to be to them (Winters, Dubuc & Higham 2015). Fantz (1958) was the first to present humans with an attention bias task in 1958 by presenting images with varying patterns and measuring the time they spent looking at each one. This bias task is also called 'visual preference task' and, if executed and interpreted properly, can be a powerful method to study a variety of questions concerning animal behaviour and cognition (Winters, Dubuc & Higham 2015). Bethell *et al.* (2012a, b) were the first to show that rhesus macaques demonstrate emotion-mediated cognitive biases comparable to other animals (see Mendl *et al.* 2009 for a review). Starlings, rats, dogs and humans have been shown to judge situations more positively following a period of enrichment or mood stimulation through music (Mendl *et al.* 2009). Bethell *et al.* (2012 a, b) reported that macaques were more likely to judge ambiguous stimuli as positive following a period of enrichment, whilst a routine health check resulted in a more negative judgement bias.

Genetic influences on cognition and behaviour in humans

A genetic polymorphism is defined as two or more alleles being present in a population with a frequency greater than 1% (Hedrick 2009). Candidate gene studies aim to identify allelic variants of certain genes that may be linked to a particular phenotype such as a certain disorder or biological pathways implicated in emotional disorders (Caspi & Moffit 2006; Canli & Lesch 2007; Dettmer & Suomi 2014). Genetic or epigenetic factors that affect the expression of a gene or its enzymatic activity can alter neurotransmission of hormones and hypothalamic-pituitary-adrenal (HPA) axis function and thereby result in variation of behavioural traits and susceptibility to stress-related neuropsychiatric disorders (Chen *et al.* 2010a), hence having an effect on the welfare of an individual.

Serotonin is a neurotransmitter with a developmental role in the brain (Todkar *et al.* 2013; Fig. 1.1) and is involved in the regulation of mood, memory and learning (Sirvio *et al.* 1994). The serotonergic system in humans has been shown to impact on controlling arousal, sleep, depression, addiction, impulsivity and anxiety (Jacobs 1991; Lesch *et al.* 1996; Owens & Nemeroff 1998; Sakado *et al.* 2003; Müller *et al.* 2007; Goenjian *et al.* 2012). The HPA system of human and non-human primates is sensitive to early experiences (Levine 1994; Capitanio *et al.* 2005) and cooperates with the serotonergic system (Barr *et al.* 2004a). Hence, serotonin helps to improve moods and to control aggression, anxiousness and impulsive behaviours (Shattuck *et al.* 2014). If underlying

genetic variants lead to a modification of serotonin signalling, it might result in behavioural variation.

Figure 1.1
Serotonin biogenesis. Serotonin is produced in the gut and in brain serotonergic neurons. The amino acid tryptophan is oxidised to 5-hydroxy-L-tryptophan. This process is catalysed by the rate limiting enzyme tryptophan hydroxylase *TPH*. 5-hydroxy-L-tryptophan is then decarboxylated to serotonin (*5-HT*). This process in turn is catalysed by aromatic L-amino acid decarboxylase (AADC). The vascular amine transporter mediates the serotonin in the presynaptic vesicules. Upon depolarization of the outer membrane of the presynaptic neuron, serotonin is brought into the synaptic cleft. In there, serotonin interacts with serotonin receptors located on the postsynaptic surface, providing neurotransmission. Neurotransmission is inhibited by receptors that reside on the presynaptic membrane, binding serotonin. The serotonin transporter (*5-HTT*) in the presynaptic membrane transfers serotonin back to the presynaptic cleft in which it is accumulated and stored until the next release. Circulating serotonin is largely derived from peripheral tissues and primarily metabolized in the liver through oxidative deamination by monoamine-oxidase A (*MAOA*). This is followed by mitochondrial aldehyde dehydrogenase (AD) which oxidises the final product of the serotonin metabolism. Polymorphic variants affecting expression and activity of *TPH*, *5-HTT* and *MAOA* also influence serotonin levels. Permission to re-use this figure was granted. Figure replicated from Pavlov, Chistiakov & Chekhonin 2012.

The serotonin or 5-hydroxytryptamine (*5-HT*) transporter (*5-HTT*) regulates the reuptake of serotonin from the synaptic cleft by transporting the *5-HT* back into the cell after its neurochemical message has been delivered (Qin *et al.* 2015; Fig 1.1). This protein is encoded by the *SLC6A4* (Solute Carrier Family 6 (Neurotransmitter Transporter)) gene. Polymorphisms in the promoter sequence of this gene (*5-HTTLPR*) have been shown to regulate the gene's expression as well as altering *in vitro* levels of transcriptional activity (Lesch *et al.* 1996; Canli & Lesch 2007). *5-HTTLPR* is an insertion/ deletion polymorphism in the promoter sequence with two predominant alleles: the long allele (l-allele) and the

short allele (s-allele), the latter is associated *in vitro* with lower quantities of *5-HTT* resulting from reduced rates of *SLC6A4* transcription (Greenberg *et al.* 1999). *SLC6A4* expression is thought to influence cortical development and hence cognitive function (Jedema *et al.* 2010). Presence of the *HTTLPR* s-allele in humans is associated with alterations in the neuroendocrine mechanisms regulating anxiety and reactivity to stress, heightened HPA axis response to stress and aversive stimuli (McCormack *et al.* 2009; Way & Taylor 2010; Qin *et al.* 2015), increased activity of the amygdala in response to emotionally relevant stimuli (Caspi *et al.* 2010), smaller left hippocampal volume (Little *et al.* 2014) and increased immune response (Fredericks *et al.* 2010). In one of the most cited studies, the s-allele has been linked to exaggeration of effects of stress in individuals exposed to increased or prolonged stress (Caspi *et al.* 2003), where s-allele carriers who had experienced childhood abuse or trauma were at higher risk for alcoholism and depression. Further, research has linked the s-allele to excessive internet use, increased smoking, alcohol abuse and pathological gambling (Lee *et al.* 2008; Feinn, Nellissery & Kranzler 2005; Lerman *et al.* 2000; Perez *et al.* 2002). Another cognitive deficit linked to the s-allele was impaired recall of a noun that preceded an emotionally valenced noun (Strange *et al.* 2008). At the same time, however, s-allele carriers also display increased creative dancing ('mankind's most ancient and universal trait reflecting a complex phenotype comprising social communication, courtship and spirituality', Bachner-Melman *et al.* 2005) and respond better to social support in the prevention and treatment of depression (Kaufman *et al.* 2004; Taylor *et al.* 2006; Brummett *et al.* 2008). Further, s-allele carriers have recently been shown to perform better in affective go/no-go tasks, an attentional test measuring an individual's ability to withhold an intentional motor response based on the valence of words (Roiser *et al.* 2007) and to be faster to learn to avoid penalizing stimuli in a passive avoidance task (Finger *et al.* 2007).

Another polymorphism that is linked to the serotonergic system is the tryptophan hydroxylase 2 (*TPH2;* Fig.1.1) insertion polymorphism (*TPH2IP*) in which 159 additional base pairs are inserted in the untranslated region (Chen *et al.* 2006, Fig. 1.2). The *TPH2* enzyme regulates serotonin by encoding tryptophan hydroxylase, which is a rate limiting enzyme in *5-HT* synthesis (Walther *et al.* 2003). In humans, *TPH2* has been linked to depression, suicide, anxiety (Zhou *et al.* 2005; Van Den Bogaert *et al.* 2006; Haghighi *et al.* 2008), bipolar affective disorder (Lopez *et al.* 2007; Cichon *et al.* 2008; Harvey *et al.* 2004) and post-traumatic stress disorder (Goenjian *et al.* 2012). *In vitro*, the short *TPH2IP* allele,

which is more common, decreases the expression of 5-*HTT*, which should theoretically increase the amount of circulating serotonin (Watson *et al.* 2015). When serotonin is increased, this should result in a downregulation or stabilization through a negative feedback loop.

Figure 1.2
Example structure of a typical human protein coding mRNA including the 5'-end and 3'-end untranslated regions. Figure adapted from Wikipedia.

The monoamine oxidase A-untranslated region variable nucleotide tandem repeat polymorphism (*MAOA-uVNTR*) also impacts on the *5-HT* pathway (Kinnally *et al.* 2010) by regulating neurotransmitter metabolism in the brain (Vanyukov *et al.* 2004; Fig. 1.1). The number of repeats in this region impacts on the production of monoamine oxidase A (*MAOA*), which is responsible for the oxidation or inactivation of the monoamines norepinephrine, dopamine and *5-HT* (Sabol *et al.* 1998; Karere *et al.* 2012). Low *MAOA* activity has been associated with impulsive behaviour, conduct disorder, aggression and impulsive violence (Gabel *et al.* 1995; Lawson *et al.* 2003; Shih *et al.* 1999; Brunner *et al.* 1993) as well as alcoholism (Hsu *et al.* 1996) and drug abuse (Gade *et al.* 1998). In humans that have experienced childhood maltreatment the low activity allele was linked to antisocial behaviour, aggression and violence (Caspi *et al.* 2002; Foley *et al.* 2004) and is thought to be linked to an impaired ability to control emotional responses during arousal (Meyer-Lindenberg *et al.* 2006).

Opioid peptides mediate natural rewards (Gianoulakis 2004). Endogenous opioids are thought to be released during infant-mother interactions, causing a stress-mitigating, rewarding effect (Weller & Feldman 2003). In humans, the μ-opioid receptor *OPRM1* is linked to nicotine use (Riju *et al.* 2011) and increased vulnerability to heroin addiction (Shi *et al.* 2002; Drakenberg *et al.* 2006). In the *OPRM1* gene, a nonsynonymous single nucleotide polymorphism (SNP) A118G, has arisen, where the G-allele causes an amino acid substitution in the N-terminal arm of the receptor, increasing their affinity for β-endorphin *in vitro* (Bond *et al.* 1998; Barr *et al.* 2007). *OPRM1* is linked to the response to negatively valenced stimuli such as physical pain (Tan *et al.* 2009), emotional rejection

(Way, Taylor & Eisenberger 2009) and stress (Hernandez-Avila *et al.* 2003). Interestingly, it was found that individuals carrying the rare G-allele show increased behavioural responses to aversive stimuli (Lee *et al.* 2011).

Dopamine is associated with regulating emotion, cognition and motor behaviour, as well as neuroendocrine signalling (Pan, Yao & Wang 2014; Fig. 1.3). The neurotransmitter dopamine affects frontostriatal circuits which subserve affective, cognitive and motor processes (Padmanabhan & Luna 2014). The dopamine transporter gene (*DAT1*) codes for carrier proteins responsible for the reuptake of dopamine from the synaptic cleft, back to the presynaptic neuron (Gizer, Fick & Waldmann 2009). Dopamine is linked to depression (Heinz *et al.* 2000; Pearson-Furhop *et al.* 2014), where lower dopaminergic neurotransmission is linked to higher risk of depression. *DAT1* influences responsiveness to reward (Dreher *et al.* 2009), aggression-related traits (Pavlov, Chistiakov & Checkhonin 2012), gambling (Comings *et al.* 2001), violent delinquency (Guo, Roettger & Shih 2007) and propensity to a criminal career (Vaughn *et al.* 2009) in humans. For *DAT1*, low dopaminergic neurotransmission makes individuals more prone to those traits.

Figure 1.3

Dopamine biogenesis. Dopamine is mainly produced by the nervous tissue and adrenal medulla from the amino acid L-tyrosine. The amino acid is hydroxylated to L-DOPA by the enzyme tyrosine hydroxylase (TH). The product is then converted to dopamine through decarboxylation by aromatic L-amino acid decarboxylase (AADC). In neurons, the vascular amine transporter transfers dopamine into vesicules that are then released into the synapse in response to a presynaptic action potential. In the synapse, dopamine binds to postsynaptic dopamine receptors (for example *DRD4*). The dopamine transporter *DAT1* mediates the reuptake of dopamine from the synapse. In several brain regions dopamine is inactivated by enzymes including monoamine

oxidase B (*MAOB*), catechol-o-methyltransferase (*COMT*) and acetaldehyde dehydrogenase to homovanillic acid. Permission to re-use this figure was granted. Figure replicated from Pavlov, Chistiakov & Chekhonin 2012.

The dopamine receptor *D4* (*DRD4*) is a member of the D2-like receptor family (Shimada *et al*. 2004; Fig 1.3). Those receptors inhibit the activity of neurons (Padmanabhan & Luna 2014) and are involved in flexible updating of information and allowing for the transition between functional states (Seamans and Yang 2004). *DRD4* is associated with novelty seeking (Ebstein *et al.* 1996), dysfunctional impulsivity (Colzato *et al.* 2010), risk-taking (Carpenter, Garcia & Lum 2011) and conduct disorder and antisocial behaviour in adolescents (Beaver *et al.* 2007), where lower dopaminergic neurotransmission makes individuals more prone to those factors. Genetic markers for the *DRD4* polymorphism in primates are still lacking and identification of further polymorphic regions in *DRD4* will contribute to our understanding of behavioural traits (Shimada *et al.* 2004).

Genetic influences on cognition and behaviour in rhesus macaques

Findings of candidate gene studies in macaques are likely to be transferable to humans due to their functionally equivalent genetic variants (Miller *et al.* 2004; Newman *et al.* 2005; Barr *et al.* 2007; Kinnally *et al.* 2010). Analogous genotypes to those mentioned in humans have also been reported in rhesus macaques (*HTTLPR*: Lesch *et al.* 1997; *TPH2*: Chen *et al*. 2006; *MAOA*: Newman *et al.* 2005; *OPRM1*: Miller *et al.* 2004; *DAT1*: Miller *et al*. 2001; *DRD4*: Livak, Rogers & Lichter 1995).

Rhesus monkeys possess a 21-base pair insertion/ deletion polymorphism orthologous to the human *5-HTTLPR* (*rh5-HTTLPR,* hereby *HTTLPR*) with two predominant alleles (Bennett *et al.* 2002): the long allele (l-allele) and the short allele (s-allele). The latter is associated with lower quantities of *5-HTT* resulting from reduced rates of *SLC6A4* transcription (Greenberg *et al.* 1999) and thought to be dominant (Kinnally *et al.* 2008). The s-allele has been linked to increased anxiety-related behaviours (Champoux *et al.* 2002), increased environmental exploration as well as self-directed behaviours following stress, increase in severity of self-directed behaviour over repeated stress exposure (Spinelli *et al.* 2012), delayed early neurobiological development, impaired serotonergic function, excessive aggression, increased HPA reactivity and heightened alcohol consumption (Barr *et al.* 2003; Barr *et al.* 2004a, b; Suomi 2006). In 2002 Bennett *et al.* showed an increase in cerebrospinal fluid (CSF) concentration, a measure of central

nervous system serotonin, was significantly influenced by *HTTLPR* genotype in monkeys that had negative early experiences (peer-rearing). Further, *5-HTTLPR* polymorphisms seem to regulate emotional behaviours and an individual's reactivity to stress (McCormack *et al.* 2009).

Importantly, most effects were only found in individuals that had early adverse experiences in the form of peer-only rearing, where they had been taken from their mother at the day of birth and brought up in groups with same-age peers only. However, in an array of cognitive tasks, the s-allele was linked to superior cognitive performance: the probability discounting task, the delay discounting tasks, the reversal learning task and the delayed match-to-sample task (Jedema *et al.* 2010). This shows that the effects of the s- and l-allele are not simply 'bad' and 'good', but much more complex.

Macaques carrying a combination of the *HTTLPR* s-allele and the *TPH2IP* (hereby *TPH2*) l-allele spent less time grooming others who in turn groomed their own partners (Brent *et al.* 2013), meaning they had fewer friends and allies. The *TPH2* gene was also linked to self-injurious behaviour and altered HPA axis function (Chen & Miller 2008; Chen *et al.* 2010a; Chen *et al.* 2010b). Further, just recently a study found that the long allele of the *TPH2* length polymorphism was linked to decreased vigilance in a social context in free-ranging macaques (Watson *et al.* 2015).

The *MAOA* polymorphism is located on the X-chromosome and consists of 5-, 6-, or 7- 18 base pair repeats in rhesus macaques (Newman *et al*. 2005). The 5 and 6 repeats confer greater transcriptional efficiency (Newman *et al*. 2005). Because females are either homozygous or heterozygous, due to the gene residing on the X-chromosome, one of which is functionally inactivated (X-chromosome inactivation) with no way to know which one (Kinnally *et al*. 2010), association studies with this gene are more complex. Hence, females homozygous for the 7 allele can be categorised as low-transcription, as can females homozygous or heterozygous for the 5 and 6 allele. However, for females who are 5-7 or 6-7 heterozygous it is unknown which one is deactivated (Newman *et al*. 2005). The low activity allele has been linked to impulsive and aggressive behaviour in peer-reared rhesus macaques as well as increased stress-reactivity (Barr *et al.* 2004c; Newman *et al.* 2005).

Reward systems such as the endogenous opioid system are critical to an individual's survival and reproduction because they are involved in driving ingestion of food, social

interactions and sexual activity (Barr & Driscoll 2014). This system is activated in response to rewards and a polymorphism in the *OPRM1* gene (C77G) results in an amino acid substitution increasing the binding affinity of the β-endorphin by the receptor, lowering blood cortisol levels and increasing aggressive threat behaviours in rhesus monkeys (Miller *et al.* 2004). The G-allele was found to be associated with differences in HPA axis output, greater levels of alcohol consumption (Barr *et al.* 2007), higher restrainment rates of infants by mothers (Higham *et al.* 2011) and increased opiate reward in response to affiliation where individuals carrying the G-allele vocalised for longer during periods of separation from their mother as well as showing higher baseline attachment behaviours (Barr *et al*. 2008).

Dopamine neurotransmission is implicated in many reward-dependent and reinforcing processes (Barr & Driscoll 2014). The *DAT1* gene in rhesus macaques has been found to be suggestive, but not predictive of hyperactivity (Miller *et al.* 2001) and Rajala *et al.* (2014) found it to be linked to impulsivity, although their result came from a study only including four males, two of which were classed as impulsive. Few studies to date have investigated the association between behaviour and *DRD4* (Coyne *et al.* 2015). A length polymorphism in the *DRD4* gene was associated with aspects of rhesus juveniles' behaviour reflecting exploration and risk-taking (Coyne *et al.* 2015) in free-ranging macaques. Those tandem repeats in the dopamine receptor *D4* gene have also been linked to physical aggression towards an unknown age- and sex-matched conspecific (Barr & Driscoll 2014). A study by Bailey and colleagues (Bailey *et al.* 2007) associated *DRD4* with novelty seeking in vervet monkeys, *Chlorocebus pygerythrus*. Additional research is needed to understand the functional significance of the *DRD4* gene in macaques (Coyne *et al.* 2015).

Importantly, studies found an additive effect of genotypes (Ferguson *et al.* 2012; Brent *et al.* 2013), where, when number of risk genotypes an individual carries increase, so does it's susceptibility to suffer from one of the various problems associated with those. Most studies found an effect of genotype only when animals had negative past experiences, mostly through peer-only or hand rearing (Kinnally *et al.* 2008; Newman *et al.* 2005). Rearing history has long been known to affect an individual's development and impact on sociality, cognition and well-being (Novak & Sackett 2006). Although such gene by environment interactions have been well recognized, the precise nature of those is still

poorly understood (Homberg & van den Hove 2012) and only recently have studies begun to explore how temperament outcomes are influenced by genetic variation that may predispose individuals to variation in sensitivity or resilience to environmental changes (Belsky *et al.* 2009). Further, many studies have been underpowered due to small sample sizes, and this study aimed to allow analysis of a comparatively large sample size.

In order to discuss impact of genotype on behavioural findings, low-expressing alleles such as the *HTTLPR* short allele have been labelled 'risk' allele (Dettmer & Suomi 2014). The validity of this labelling will be discussed further.

Attention bias

Understanding genetic variants that influence stress reactivity would aid in prediction of whether an individual is at increased risk for psychopathology (Caspi *et al.* 2002, 2003) and a good way to assess this is by exploring and evaluating how an individual responds to environmental stress, how strongly and how long it reacts and what kinds of responses it exhibits following exposure to stress (Barr & Driscoll 2014).

Attention bias has only recently been studied in rhesus macaques (Bethell *et al.* 2012a, b; King *et al.* 2012; Lacreuse *et al.* 2013; Mandalaywala, Parker & Maestripieri 2014). Male rhesus macaques (King *et al.* 2012; Lacreuse *et al.* 2013) and infants at the age of nine months (Mandalaywala, Parker & Maestripieri 2014) have been shown to exhibit increased vigilance towards threatening faces of conspecifics. Further, emotion has been found to mediate attention (Bethell *et al.* 2012a, b), where male rhesus macaques that recently underwent invasive health checks were more avoidant of aggressive conspecific facial expressions compared to macaques which had received enrichment, indicating a difference in internal state, and hence emotion, in those two groups.

Whether this attention bias is further influenced by underlying genetic traits is unknown. Studying attention bias in relation to an individual's genotype would therefore give way to exploration of underlying factors of cognitive bias and how an individual's stress response is influenced by its genotype. I hypothesized that attention bias for social and emotional stimuli would be influenced by genotype. Due to the many impacts on reward-related behaviours, I hypothesized that genotype would also have an impact on how well an individual responds to habituation to aversive stimuli.

In order to assess whether genotype has an impact on attention bias, this study had several aims. Aim 1 was to assess current published protocols used for genotyping of specific polymorphisms and whether they produce reliable results. Aim 2 was to identify if and how genotype affects attention bias towards unknown conspecific stimuli (social stimuli) as well as behaviour after having been presented with those stimuli (Experiment 1). Aim 3 was to assess if and how genotype affects attention bias towards a known, thought to be aversive, human stimulus, habituation to a known, and thought to be aversive human stimulus and behaviour after having been presented with those stimuli (Experiment 2).

In order to meet Aim 1 current methods of genotyping for known genetic polymorphisms were replicated. Published protocols should be easy to follow and produce reliable results, hence allow for the genotyping of the here studied rhesus macaques. If those methods are not reliable, new primers will be designed to allow for precise genotyping. Those objectives were investigated in Chapter 2.

Aim 2 was to investigate whether the genotypes had an impact on attention bias. Specifically, the *HTTLPR* s-allele seems to increase the risk of developing psychopathology, particularly in the context of stress (Barr & Driscoll 2014) and should lead to increased vigilance for threat. The *TPH2* l-allele has been found to have an additive effect in combination with the *HTTLPR* s-allele (Brent *et al.* 2013) and those genotypes combined could lead to findings of their impact on attention bias. As the low activity *MAOA* allele is thought to lead to impaired ability to control emotional responses during arousal (Meyer-Lindenberg *et al.* 2006), it is likely that, during presentation of emotionally loaded stimuli, an individual will respond in a different, possibly stronger way compared to individuals that do not carry the low activity allele. As the G-allele of the *OPRM1* gene has been found to increase aggressive threat behaviours in macaques (Miller *et al.* 2004) and to increase behavioural responses to aversive stimuli in humans (Lee *et al.* 2011) it might also have an effect on attention bias towards aggressive and threatening stimuli as well as behaviour. Further, as *DAT1* has been found to be linked to responsiveness to reward and aggression-related traits (Dreher *et al.* 2009; Pavlov, Chistiakov & Checkhonin 2012), *DAT1* could also have an impact on attention bias towards stimuli that might have different emotional valence and bring with them possible advantages or disadvantages of looking at them. Lastly, as *DRD4* was associated with risk taking in rhesus juveniles (Coyne

et al. 2015) and novelty-seeking in a different primate species (Bailey *et al.* 2007) it might also be associated with bias towards a stimulus which might present a risk to an individual when direct eye contact is made. Experiment 1 investigated this aim in Chapter 3.

Aim 3 investigated whether genotype has an effect on the success to habituate an individual to aversive stimuli. Specifically individuals carrying the *OPRM1* G-allele should respond better to the rewarding effect of habituation (Barr *et al.* 2008). Individuals carrying the low activity *MAOA* 7-repeat allele should respond less well, due to the impaired ability to control emotional responses in humans (Meyer-Lindenberg *et al.* 2006) and the *DRD4* genotype could influence responsiveness to habituation if it is linked to novelty-seeking in rhesus macaques. Chapter 4 presents the investigation into this aim with Experiment 2.

The larger the sample size, the more likely it should be to detect an effect of genotype (Sorenson *et al.* 2012) and this study provides the basis to explore the effect of genotype on attention bias in a relatively large sample. Additionally, parallel findings between human and non-human primate studies strengthen the validity of findings supporting a relationship between the studied variables (Sorenson *et al.* 2012). Hence, this study aims to replicate the findings of human studies where *HTTLPR* genotype impacts on attention bias, it would be an important indicator to the validity of all those studies. The likelihood of only one gene impacting attention bias is small and hence a variety of candidate genes that might have an impact on attention bias were included.

Chapter 2. Genetical analysis and validation of genotyping methods

This chapter gives a short introduction to the different procedures to analyse DNA that were carried out for the purpose of this study. Protocols to genotype DNA were those that are published in current literature. In order to validate those metods, I replicated the exact procedures. The methods present the procedures which were used to genotype 65 female rhesus macaques for the genotypes investigated in this stuy. I discuss the way DNA samples were analysed and genotypes were assigned to each individual. It contains a variety of methods trialled for analysis as well as the final method used for analysis and the achieved results. The discussion of this chapter focuses on the allele frequencies and how those fit into the broader picture as well as the problems encountered when published methodologies were followed. I conclude that many published protocols were not possible to be replicated in this study and need to be refined further.

Genetics introduction

The polymerase chain reaction (PCR), is used to amplify large quantities of specific sections of DNA for further study. In this process deoxyribonucleic acid (DNA) is denatured into single-stranded DNA. Then, using those strands as templates, DNA polymerase builds two new strands of DNA, a process that is repeated several times (McPherson & Møller 2006). By using specific primers particular regions of DNA of interest can be targeted. The amplified product then can be separated and size fractionated through agarose gel electrophoresis. Length polymorphisms contain insertion/deletion segments which differ in their size. As the deletion segments are smaller in size (containing less base pairs), they travel through the gel comparatively quicker than those containing the insertion. By placing a ladder containing segments with set amounts of base pairs (i.e. 100bp, 200bp, 300bp …) the bands on the gel can be scored in base pair size.

Single nucleotide polymorphisms (SNPs) vary at just one base but do not differ in length (Lesk 2012). In order to visualise differences and score genotypes of individuals there are a variety of options though two are commonly utilised. First, restriction enzymes specific to the region containing the SNP can be used. When the enzyme recognition sequence spans the SNP then restriction fragment length polymorphisms can be scored via electrophoresis. Secondly, a Taqman assay can be used for genotyping. In this process

two probes are designed each complementary to the two alternative alleles. Each probe is labelled with a specific fluorescent tag, and at the other end of the probe a quencher molecule quenches the fluorescence of the labelled tag. During PCR the probe is incorporated into the PCR product, the quencher is released and the fluorescence can be measured (Fig 2.1).

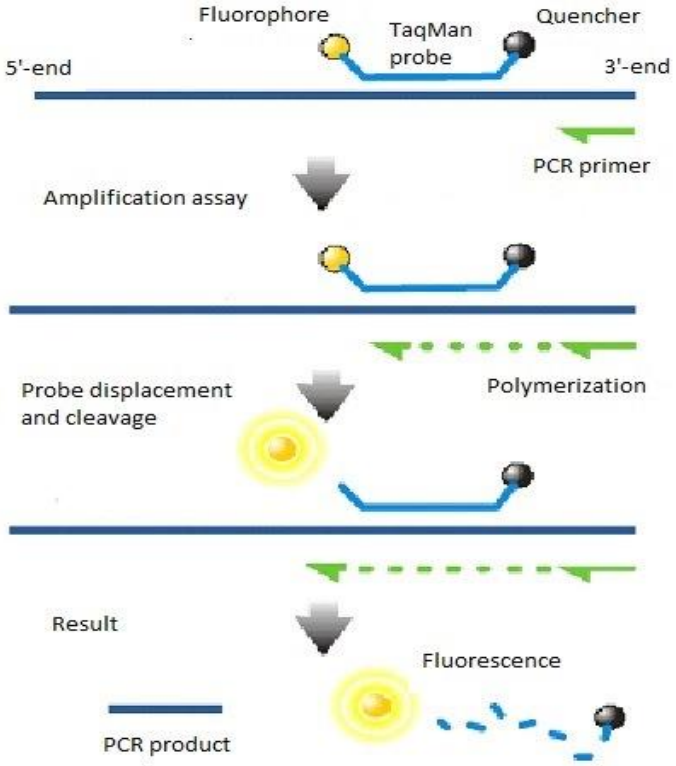

Figure 2.1
Example of the functioning of a TaqMan probe. The probe consists of a quencher (attached at the 3'-end) and a fluorophore (attached to the 5'-end). The quencher, as long as it is in proximity to the fluorophore, quenches the fluorescence of it. The probe anneals to its specific DNA target which is amplified by a specific set of primers. When the primer synthesizes the DNA strand, the probe is incorporated into the PCR product and the quencher is then released removing it from proximity to the fluorophore. The PCR cycler can now detect fluorescence in the PCR product. Since probes are allele specific the intensity of fluorescence of each probe can be used to determine genotype. Figure adapted from Wikipedia.

Materials and Methods

2.1 DNA collection

DNA of sixty-five female rhesus macaques was analysed for this study, of which twenty nine took part in the attention bias study. Blood samples were collected by a veterinarian into EDTA K2 (anticoagulant) tubes whilst the animals were sedated during their routine health check. The blood sample tubes were wrapped in cotton wool and sent to B&K Universal Laboratories (Grimston, East Yorkshire, UK) the day after collection. DNA was extracted on the day of arrival using the DNeasy blood and tissue kit from Qiagen (Catalogue no. 69506) and concentrations were measured using a NanoDropLite (ThermoFisher, UK). DNA was transported to Liverpool John Moores University, Byrom Street Campus and stored at -20°C short-term until the first use and at 4°C during routine use.

2.2 Genotyping

Both length variants and single nucleotide polymorphisms (SNPs) that could be tested for association with attention bias were identified through genotyping. DNA was genotyped for the following known polymorphisms: length polymorphisms in *(a)* the promoter region of the gene encoding the serotonin transporter (5-hydroxytryptamine transporter, 5-*HTTLPR*), *(b)* monoamine oxidase A (*MAOA)*, and *(c)* tryptophan hydroxylase 2 (*TPH2)* as well as a single nucleotide polymorphism (C77G) in the mu-opioid receptor (*OPRM1)*. Additionally, amplification and sequencing of segments of both the dopamine transporter (*DAT1)* and dopamine receptor D4 (*DRD4)* was attempted in order to identify novel polymorphisms. Since a variety of published primer pairs and amplification procedures are available for these loci, preliminary studies were carried out to optimise PCR reactions and find the most suitable primer pairs for routine testing (Table 2.1; *HTTLPR*: Table 2.2, *TPH2*: Table 2.3, *MAOA*: Table 2.4, *OPRM1*: Table 2.5, *DAT1*: Table 2.6, *DRD4*: Table 2.7). Where previously designed primers did not yield results, new primers were designed for the purpose of this study.

Table 2.1. Primer pairs used for preliminary analysis and source of those that were taken from the existing literature. Some primers were newly designed for the purpose of this study and are indicated by the following sign: /.

Gene	Primer pairs	Source of primer pair or newly designed primer (/)
HTTLPR	HTTLPR_stpr5 (5'-GGCGTTGCCGCTCTGAATGC-3') HTTLPR_intl (5'-CAGGGGAGATCCTGGGAGGG-3')	Bennett *et al.* 2002 Bennett *et al.* 2002
	5HTT-F (5'-GCCGCTCTGAATACCAGCAC-3') 5HTT-R (5'-GGAGGGATGCAGGGGTTG-3')	Karere *et al.* 2012 Karere *et al.* 2012
	STR-R1 (5'-GAGGGACTGAGCTGGACAACCAC-3') HTTLPR_stpr5 (5'-GGCGTTGCCGCTCTGAATGC-3')	Kinnally *et al.* 2008 Bennett *et al.* 2002
	OLER-F (5'-CAGCACCTAACCCCCTAATGCCCTG-3') OLER-R (5'-GATTCTGGTGCCACCTAGACGCCAG-3')	Rogers *et al.* 2006 Rogers *et al.* 2006
	5HTT-R (5'-GGAGGGATGCAGGGGTTG-3') OLER-F (5'-CAGCACCTAACCCCCTAATGCCCTG-3')	Karere *et al*. 2012 Rogers *et al.* 2006
	HTTLPR_intl(5'-CAGGGGAGATCCTGGGAGGG-3') OLER-F (5'-CAGCACCTAACCCCCTAATGCCCTG-3')	Bennett *et al*. 2002 Rogers *et al.* 2006
	5HTT-R (5'-GGAGGGATGCAGGGGTTG-3') HTTLPR_stpr5 (5'-GGCGTTGCCGCTCTGAATGC-3')	Kinnally *et al.* 2008 Bennett *et al.* 2002
	5HTT-F (5'-GCCGCTCTGAATACCAGCAC-3') HTTLPR_intl (5'-CAGGGGAGATCCTGGGAGGG-3')	Karere *et al.* 2012 Bennett *et al.* 2002
TPH2	TPH2-U3F5 (5'-TGTAGGAAACTTCTCATCACAA-3') TPH2-U3R5 (5'-CAGCATAAAATTCATAGTCCCAAG-3')	Chen *et al*. 2006 Chen *et al.* 2006
MAOA	MAOA-F (5'-CCCAGGCTGCTCCAGAAAC-3') MAOA-R (5'-GGACCTGGGAAGTTGTGC-3')	Newman *et al.* 2005 Newman *et al.* 2005
	MAOA-F(2) (5'-CAGAAACATGAGCACAAACG-3')	Sullivan, Mendoza & Capitanio 2011
	MAOA-R(2) (5'-TACGAGGTGTCGTCCAAGTT-3')	Sullivan, Mendoza & Capitanio 2011
OPRM1	OPRM1_C77G_F (5'- TGGCGCACTCAAGTTGCT-3') OPRM1_C77G_R (5'- GGGACAAGTTGACCCAGGAA-3') (probe OPRM1_C77G_VIC (5'-CAGCACGCAGCCC-3') labelled with VIC at 5'end for detection of the G allele probe OPRM1_C77G_FAM (5'-CAGCACCCAGCCC-3') labelled with 6-FAM for detection of the C allele)	/ /
DAT1	DAT1-3'R1 (5'-TGCTCTTACTCATGGGCACA-3') DAT1-3'F1 (5'-AGCACTTGAACCCAGGAGTT-3')	/ /
	DATR1 (5'-GACTGTTGGCGACTTTGGA-3')	Miller-Butterworth *et al.* 2008
	DAT1-3'F2 (5'-ATGGTTTTCTCATGCGACCG-3')	/

DRD4	DRD4EX3F (5'-GCGACTACGTGGTCTACTCG-3')	Ebstein *et al.* 1996
	DRD4-EX3R (5'-AGGACCCTCATGGCCTTG-3')	Ebstein *et al*. 1996
	DRD4-PROM-RPT-F (5'-GGGAGGATGAGGCCAGAGAAT-3')	/
	DRD4-PROM-RPT-R (5'-GAAGGAGCAGGCACCGTGAGC-3')	Seaman *et al.* 1999
	DRD4-PROM-SNP-F (5'-CGGGGGCTGAGCACCAGAGGCTGCT-3')	Okuyama *et al.* 2000
	DRD4-PROM-SNP-R (5'-GCATCGACGCCAGAGCCATCCTGCC-3')	Okuyama *et al.* 2000

Table 2.2. PCR conditions for optimisation of *HTTLPR* amplification. Primers and PCR conditions used for final genotyping are highlighted in grey.

PCR cycles and temperatures	Primer
95°C for 5mins, 40 cycles at 95°C for 30s, 61°C for 30s, 72°C for 1min, followed by a final extension at 72°C for 7mins	5HTT-R and HTTLPR_stpr5 ,
	5HTT-R and OLER-F
	HTTLPR_intl and OLER-F
	5HTT-F and HTTLPR_intl
95°C for 3 mins, 35 cycles at 94°C for 30s, 63°C for 30 s, 72°C for 30s,followed by a final extension at 72°C for 5mins	HTTLPR_stpr5 and HTTLPR_intl
95°C for 3 mins, 35 cycles at 94°C for 30s, 67°C for 30 s, 72°C for 30s,followed by a final extension at 72°C for 5min (Bennett *et al.* 2002)	HTTLPR_stpr5 and HTTLPR_intl
95°C for 3 mins, 35 cycles at 94°C for 30s, 70°C for 30 s, 72°C for 30s,followed by a final extension at 72°C for 5min	HTTLPR_stpr5 and HTTLPR_intl
96°C for 5 mins, 30 cycles at 94°C for 15s, 60°C for 15s, 72°C for 30s,followed by a final extension at 72°C for 3min (Barr *et al.* 2004b)	HTTLPR_stpr5 and HTTLPR_intl
95°C for 5 mins, 40 cycles at 94°C for 1min, 55°C for 1min, 72°C for 1min, followed by a final extension at 72°C for 10mins (Karere *et al.* 2012)	5HTT-F and 5HTT-R
95°C for 5 mins, 35 cycles at 95°C for 30s, 52°C for 30s, 72°C for 30s, followed by a final extension at 72°C for 5mins (Kinnally *et al*. 2008)	STR-R1 and HTTLPR_stpr5
96°C for 4 mins, 37 cycles at 94°C for 40s, 61°C for 30s, 72°C for 30s, followed by a final extension at 72°C for 7min (Rogers *et al.* 2006)	OLER-F and OLER-R
95°C for 5mins, 40 cycles at 95°C for 30s, 61°C for 30s, 72°C for 1min, followed by a final extension at 72°C for 7mins (Brent *et al.* 2013, Athy Robinson *pers. comm*.)	5HTT-F and 5HTT-R

Table 2.3. PCR conditions for optimisation of *TPH2* amplification. Primers and PCR conditions used for final genotyping are highlighted in grey.

PCR cycles and temperatures	Primer
95°C for 3 mins, 35 cycles at 94°C for 30s, 55°C for 30 s, 72°C for 30s and a final extension at 72°C for 5mins	TPH2-U3F5 and TPH2-U3R5

Table 2.4. PCR conditions for optimisation of *MAOA* amplification. Primers and PCR conditions used for final genotyping are highlighted in grey.

PCR cycles and temperatures	Primer
95°C for 5mins, 40 cycles at 94°C for 30s, 55°C for 30 s, 72°C for 30s and a final extension at 72°C for 10mins	MAOA-F(2) and MAOA-R(2)
95°C for 3 mins, 35 cycles at 94°C for 30s, 65°C for 30 s, 72°C for 30s and a final extension at 72°C for 5mins	MAOA-F and MAOA-R
95°C for 3 mins, 35 cycles at 94°C for 30s, 67°C for 30 s, 72°C for 30s and a final extension at 72°C for 5mins	MAOA-F and MAOA-R
95°C for 5 mins, 40 cycles at 94°C for 1min, 55°C for 1min, 72°C for 1min and a final extension at 72°C for 10mins (Sullivan, Mendoza & Capitanio 2011)	MAOA-F(2) and MAOA-R(2)

Table 2.5. PCR conditions for Taqman genotyping of the C77G polymorphism in *OPRM1* amplification. Primers and PCR conditions used for final genotyping are highlighted in grey.

PCR cycles and temperatures	Primer
95°C for 15mins, 40 cycles at 92°C for 15s and 60°C for 1min	OPRM1_C77G_F and OPRM1_C77G_R

Table 2.6. PCR conditions for optimisation of *DAT1*. None of those were included in the final analysis.

PCR cycles and temperatures	Primer
95°C for 5mins, 35 cycles at 94°C for 30s, 57°C for 30 s, 72°C for 30s and a final extension at 72°C for 5mins	DAT1-3'R1 and DAT1-3'F1
95°C for 5mins, 35 cycles at 94°C for 30s, 57°C for 30 s, 72°C for 30s and a final extension at 72°C for 5mins (Miller-Butterworth *et al.* 2008)	DATR1 and DAT1-3'F2
95°C for 5mins, 36 cycles at 94°C for 30s, 60°C for 30 s, 72°C for 1min and a final extension at 72°C for 5mins	DAT1-3'R1 and DAT1-3'F1

Table 2.7. PCR conditions for optimisation of *DRD4* amplification. Primers and PCR conditions used for final genotyping are highlighted in grey.

PCR cycles and temperatures	Primer
95° for 1min, 35 cycles at 95° for 20s, 72°C for 30s and a final extension at 72°C for 7 mins	DRD4-PROM-SNP-F and DRD4-PROM-SNP-R
95° for 3mins, 35 cycles at 94° for 30s, 60°C for 30s, 72°C for 1 min and a final extension at 72°C for 7 mins	DRD4EX3F and DRD4-EX3R
95° for 3mins, 35 cycles at 94° for 30s, 60°C for 30s, 72°C for 1 min and a final extension at 72°C for 7 mins	DRD4-PROM-RPT-F and DRD4-PROM-RPT-R
95° for 3mins, 35 cycles at 94° for 30s, 62°C for 30s, 72°C for 1 min and a final extension at 72°C for 7 mins	DRD4-PROM-RPT-F and DRD4-PROM-RPT-R
98° for 1min, 35 cycles at 98° for 20s, 68°C for 30s, 72°C for 2 min and a final extension at 72°C for 3mins	DRD4EX3F and DRD4-EX3R
98° for 1min, 35 cycles at 98° for 20s, 68°C for 30s, 72°C for 20s and a final extension at 72°C for 3mins	DRD4EX3F and DRD4-EX3R
95° for 5min, 35 cycles at 94° for 30s, 58°C for 30s, 72°C for 20s and a final extension at 72°C for 3mins	DRD4EX3F and DRD4-EX3R

2.3 Design of Taqman assay

A novel Taqman assay for genotyping the *OPRM1* C77G polymorphism was designed using the Custom TaqMan Assay Design Tool (https://www.lifetechnologies.com/order/custom-genomic-products/tools/genotyping/). Briefly, the sequence surrounding the SNP (100 base pairs flanking either side) was submitted for assay design:
GGTGCCCGGCCGGCCGTCAGTACCATGGACAGCAGCGCTGTCCCCACGAACGCCAGCAATTGCA CTGATGCCTTGGCGCACTCAAGTTGCTCCCCAGCAC[C/G]CAGCCCCGGTTCCTGGGTCAACTTGT CCCACTTAGATGGCAACCTGTCCGACCCATGCGGTCCGAACCGCACCGACCTGGGCGGGAGAGA CAGCCTGTGC

Forward and reverse primers and two minor groove binding (MGB) probes (Applied Biosystems) were designed using the custom design tool. In this Taqman assay (assay ID AH399N0) OPRM1_C77G_F (5'- TGGCGCACTCAAGTTGCT-3'), and OPRM1_C77G_R (5'-GGGACAAGTTGACCCAGGAA-3') were standard oligonucleotides with no modification. The probe OPRM1_C77G_VIC (5'-CAGCACGCAGCCC-3') was labelled with VIC at the 5'end for the detection of the G allele and the probe OPRM1_C77G_FAM (5'-CAGCACCCAGCCC-3') was labelled with 6-FAM for detection of the C allele. Each probe also had a 3' non-fluorescent quencher and a minor groove binder at the end (see Fig. 2.1). The minor groove binder provides more accurate allelic discrimination by increasing the T_m (melting temperature) between matched and mismatched probes.

2.4 Polymerase Chain Reactions (PCRs)

PCR procedures followed standard methods (see for example McPhersin & Møller, 2006). All PCR amplifications (except *OPRM1)* were undertaken on a Bio Rad T100 ™ Thermal Cycler and products visualised following agarose gel electrophoresis in a Bio Rad Sub-Cell® GT Agarose Gel Electrophoresis Systems with BioRad Power Pac™ HC Power Supply. Agarose gels (1.5- 3.5%, Appendix 1) were prepared using NuSeive agarose (Thermo Scientific, TopVision agarose) in 1xTBE buffer (diluted 10xTBE buffer from Thermo Scientific) with 5µl GelRed DNA stain. Electrophoresis was conducted at 100 Volt and products finally examined under UV light using a BioRad GelDoc XR system and sized versus a 100bp DNA ladder (ThermoFisher). All samples for *HTTLPR*, *TPH2* and *MAOA*

were scored twice for each genotype and the few remaining inconsistent results were scored again.

HTTLPR amplification was undertaken in 25µl reaction volume consisting of 12.5µl PCR mastermix (GoTaq Hot Start Colorless Master Mix, Promega), 9.5µl water, 1µl primer 5HTT-R, 1µl primer HTTLPR_stpr5 and 1µl DNA. The PCR product was examined on a 2% NuSeive agarose gel (Thermo Scientific, TopVision agarose) for one and a half hours.

MAOA amplification was undertaken in 25µl reaction volume consisting of 12.5µl PCR mastermix (2x VWR Taq Master Mix (containing 1.5mM $MgCl_2$)), 9.5µl water, 1µl primer MAOA-R, 1µl primer MAOA-F and 1µl DNA. The PCR product was then electrophoresed on a 3.5% NuSeive agarose gel for two hours.

The 25µl *TPH2* PCR reaction consisted of 12.5µl PCR mastermix (2x VWR Taq Master Mix (1.5mM $MgCl_2$)), 9.5µl water, 1µl primer TPH2-U3F5, 1µl primer TPH2-U3R5 and 1µl DNA. PCR products were run for one hour on a 1.5% NuSeive agarose gel (Thermo Scientific, TopVision agarose).

OPRM1 was genotyped using the custom Taqman genotyping assay run on a 96 well plate with optical caps. The reaction mix consisted of 2µl 5x qPCRmix (HOT FIREPol® Probe qPCR Mix Plus from Solis Biodyne), 0.25µl primer probe (OPRM1_C77G from Life Technologies), 6.75µl water and 1µl of DNA. Genotyping was undertaken on a Stratagene MX3005 real time PCR machine with one cycle of 95°C for 15mins, followed by 40 cycles of 92°C for 15s and 60°C for 1 min. The machine read the fluorescence in the VIC and FAM channels and genotypes were automatically assigned from endpoint fluorescence data.

Amplification of *DAT1* was conducted in a 25µl reaction (12.5µl PCR mastermix (2x VWR Taq Master Mix (1.5mM $MgCl_2$)), 9.5µl water, 1µl primer DAT1-3'R1, 1µl primer DAT1-3'F1, 1µl DNA). Following visualization of PCR products in a 1.5% agarose gel, reaction products were purified using the GeneJETPCR Purification Kit from Fisher (Appendix 1 for exact procedure) and sent off for sequencing (GATC Light Run Sequencing, GATC Biotech).

DRD4 was amplified in a 25µl reaction consisting of 12.5µl PCR mastermix (2x VWR Taq Master Mix, (1.5mM $MgCl_2$)), 9.5µl water, 1µl primer DRD4-PROM-SNP-F, 1µl primer DRD4-PROM-SNP-R and 1µl DNA. Successful reactions were purified using the GeneJET PCR Purification Kit from Fisher (Appendix 1 for exact procedure) and sent off for sequencing using Light Run Sequencing vouchers from GATC Biotech.

All laboratory work was carried out by myself under supervision of Dr Craig Wilding, whilst DNA sequencing was done by GATC Biotech who provide the sequences online once they have sequenced all samples. Sequences were then analysed using CodonCode Aligner (CodonCode Corporation) and SNPs identified following alignment with ClustalX (Larkin *et al.* 2007).

Results

2.5 PCR results

Eight different primer pairs were trialled for the amplification of *HTTLPR* using a variety of published protocols (Table 2.2). There was a wide variety in amplification success with a range of outcomes including failed PCRs, non-specific banding and faint results that were not fit for interpretation. The optimum primer pair and method followed Brent *et al.*'s (2013) amplification protocol using a combination of primers from Bennett *et al.* (2002) and Kinally *et al.* (2008). Using this method, all 65 females were genotyped for *HTTLPR* and genotype frequencies are presented in Table 2.8. Figure 2.2 shows an example of some of the *HTTLPR* samples on an agarose gel under UV light.

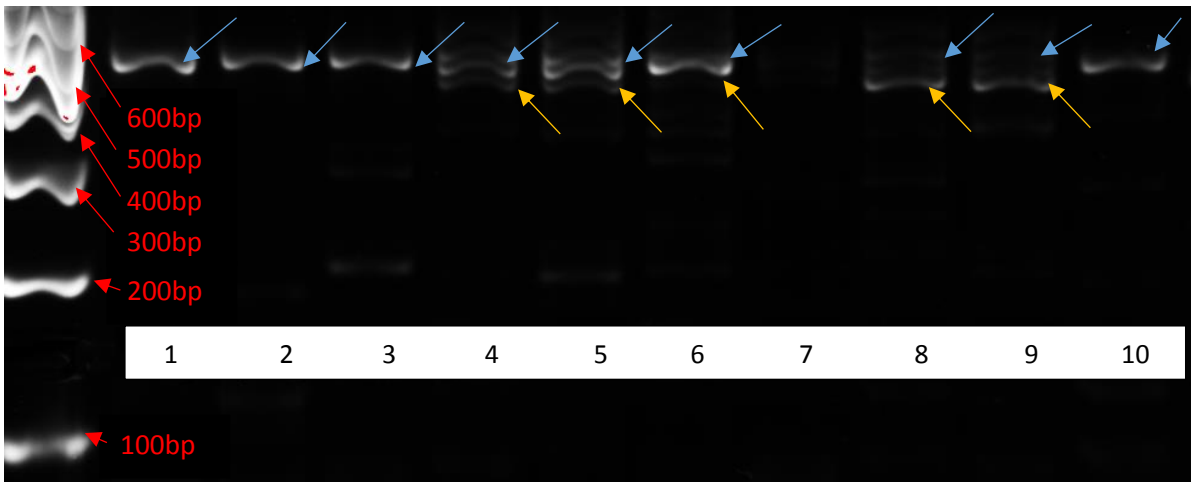

Figure 2.2
PCR products from *HTTLPR* gel electrophoresis of eleven samples of female rhesus macaques, *Macaca mulatta*. Samples 1, 2, 3 and 10 were l-homozygous, 4,5,6,8 and 9 were heterozygous (as confirmed through repeated testing) and sample 7 was unsuccessful. Blue and yellow arrows mark the long and short allele respectively and numbers indicate lanes for each sample. Ladder is marked in red.

Two different primer pairs were trialled for amplification of *MAOA* using four different protocols (Table 2.4). The outcome included unclear and faint bands for three of those protocols. The primers used for analysis came from Sullivan *et al*. (2011) with adaptations. Using this method, all 65 macaques were genotyped (Table 2.8). Although some additional bands were visible in some samples, bands of sizes consistent with the expected sizes of the 5, 6 and 7 repeat alleles were clearly scorable and additional bands were not scored. An example of the *MAOA* bands under UV light can be seen in Figure 2.3.

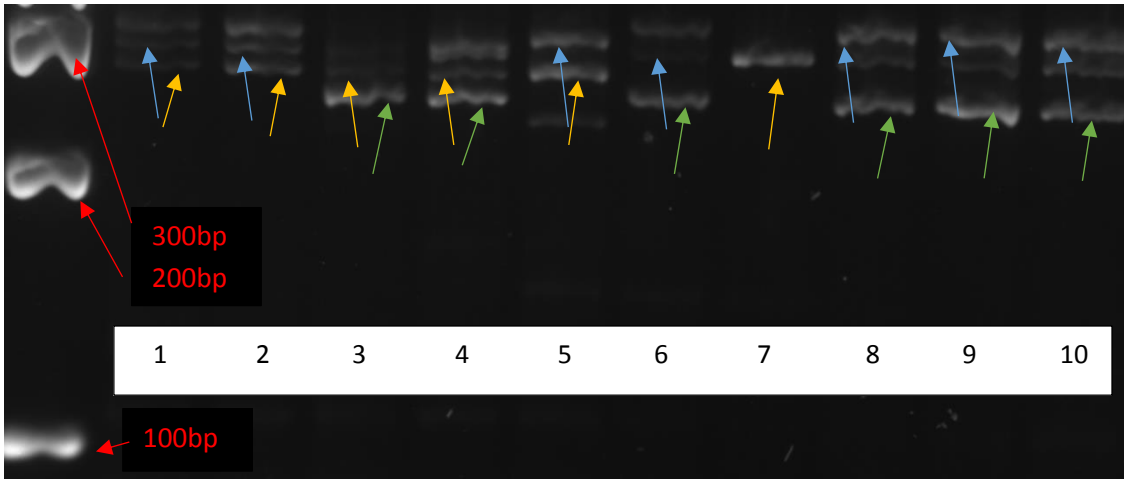

Figure 2.3
PCR products from *MAOA* gel electrophoresis of ten samples of female rhesus macaques, *Macaca mulatta*. Samples 1, 2 and 5 were 6-7 repeats, 3 and 4 were 5-6 repeats, 6, 8, 9 and 10 were 5-7 repeats and sample 7 was 6-6 repeats. Blue arrows point to 7-repeats, yellow to 6-repeats and green to 5-repeats. Ladder is annotated in red and numbers indicate lanes for each sample.

The Taqman assay for analysis of *OPRM1* yielded clear results (Table 2.5). Genotype frequencies are presented in Table 1. Figure 2.4 shows an example output from the real time PCR machine displaying relative fluorescence of samples.

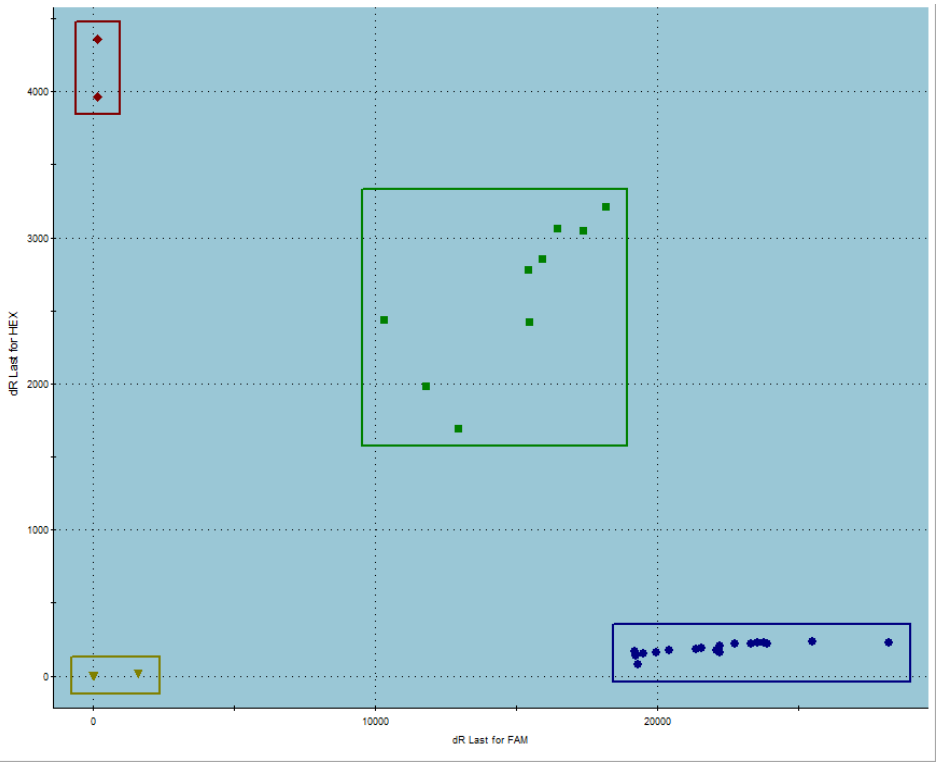

Figure 2.4
Output given by the real time PCR machine for the *OPRM1* C77G genotype. Red dots represent individuals homozygous for the G-allele, blue dots represent individuals homozygous for the C-allele and green dots represent heterozygous individuals (CG). The machine additionally produces an output linking each well to its specific genotype.

The primer pair and protocol for *TPH2* (Table 2.3) yielded consistent, clear results and all 65 samples were scored using those methods (Table 2.8). Examples of some of the *TPH2* samples on an agarose gel under UV light can be seen in Figure 2.5.

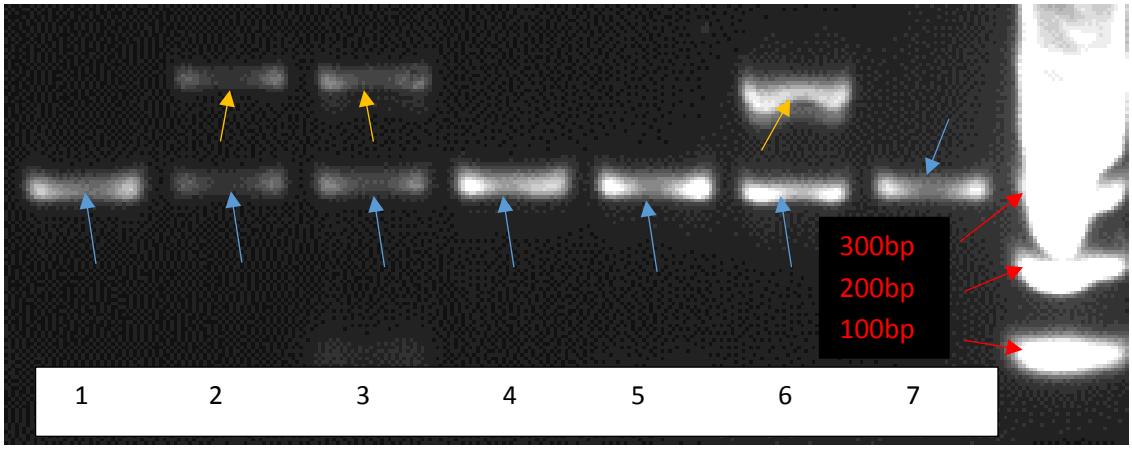

Figure 2.5
PCR products from *TPH2* gel electrophoresis of eleven samples of female rhesus macaques, *Macaca mulatta*. Samples 1, 4, 5 and 7 were s-homozygous and samples 2, 3 and 6 were heterozygous. Blue arrows indicate short alleles and yellow arrows indicate long alleles. Ladder is marked in red and numbers indicate lanes for each sample.

Again, a variety of different primer pairs were trialled for the amplification of *DRD4* using a variety of published protocols (Table 2.7). There was a wide variety in amplification success with a range of outcomes including failed PCRs, non-specific banding and faint results that were not fit for interpretation. The optimum primer pair and method followed an optimised amplification protocol using primers from Okuyama *et al.* (2000). Figure 2.6 shows an example of some of the *DRD4* bands in samples that were purified and sent for sequencing.

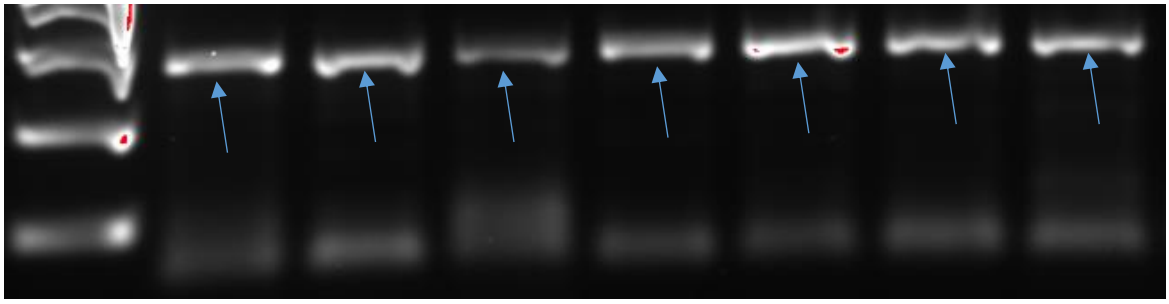

Figure 2.6
PCR products from *DRD4* gel electrophoresis of seven samples of female rhesus macaques, *Macaca mulatta*. Samples yielded bands and were sent off for sequencing. Blue arrows indicate bands.

Although the PCR product yielded light bands (Table 2.6), sequencing of *DAT1* was unsuccessful and due to limited time this locus was therefore not included in further analysis.

Genotype frequencies for all 65 genotyped females can be found in Table 2.8. Frequencies of the 29 individuals that took part in the attention bias studies are also presented as well as frequencies in other studies. A list of each individual's genotype can be found in Appendix 2.

Table 2.8. Genotype frequencies for loci analysed in this study for the whole data set of female rhesus macaques, *Macaca mulatta* (n=65). Genotype frequencies of the 29 females that took part in the attention bias studies are also presented, as well as genotype frequencies of other studies.

Gene	Genotype frequencies for 65 females	Genotype frequencies for 29 females	Genotype frequencies from other studies
HTTLPR	LL: 46.15% SL: 49.23% SS: 4.62%	LL: 44.83% SL: 48.28% SS: 6.9%	(Barr *et al.* 2013: LL: 50.5%, SL: 39.3%, SS: 8.4%; Karere *et al.* 2012: LL: 70.27%%, SL: 5.41%, SS: 21.62%; and Ferguson *et al.* 2012: LL: 67.57%, SL: 29.73%, SS: 2.7%)
TPH2	SS: 69.23% SL: 26.15% LL: 4.62%	SS: 72.41% SL: 24.14% LL: 3.45%	(Barr *et al.* 2013: SS: 52.3%, SL: 43%, LL: 4.7%)
MAOA	55: 9.38% 56: 4.69% 66: 10.94% 57: 29.69% 67: 26.56% 77: 18.75%	55: 10.34% 56: 3.45% 66: 17.24% 57: 27.59% 67: 31.03% 77: 10.34%	(Karere *et al.* 2012: 55: 10.53%, 56: 21.1%, 66: 10.53%, 57: 31.58%, 67: 10.53%, 77: 15.79%)
OPRM1	CC: 80% CG: 16.92% GG: 3.1%	CC: 86.21% CG: 13.79% GG: 0%	(Higham *et al.* 2011 : CC: 43.76%, CG: 46.87%, GG: 9.37%; Barr *et al.* 2008: CC: 71.88% , CG: 27.1%, GG: 0.01%; Miller *et al.* 2004: CC: 43.75, CG: 50%, GG: 6.25%; and Ferguson *et al*. 2012: CC: 70.27%, CG: 29.73%, GG: 0%)

2.6 DNA sequencing

DRD4 PCR products from sixty five female rhesus macaques were sequenced. Table 2.9 shows the SNPs identified and allele frequencies for the studied population. A full list of genotypes of each study subject can be found in the appendix (Appendix 2). Sequencing of the *DRD4* PCR product showed several novel SNPs (Fig. 2.7, Table 2.9).

Table 2.9. Novel SNPs from *DRD4* sequencing.

Chromosome	Position	Reference genome base	Alternative base	Allele frequency of reference base	Allele frequency of alternative base
14	447934	G	T	95.5%	4.5%
14	448009	A	T	65.38%	34.62%
14	448055	G	C	92.31%	7.69%
14	448080	G	C	94.62%	5.38%
14	448097	G	A	94.62%	5.38%

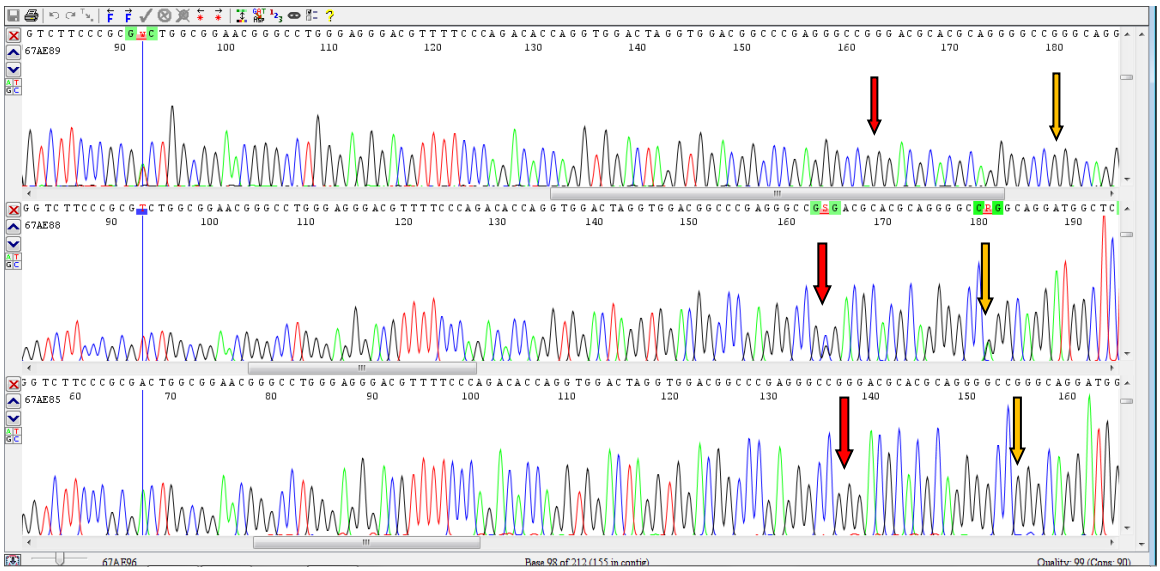

Figure 2.7
Screenshot from CodonCode Aligner showing chromatograms from three female rhesus macaques, *Macaca mulatta*, with three variable SNPs highlighted. The A>T SNP at 14:448009 is indicated by the vertical line. The upper panel is an AT heterozygote, the middle panel a TT homozygote and the lower panel an AA homozygote. Two other SNPs (14:448080 G-C and 14:448097 G-A) are indicated with red and orange arrows respectively. (Note that due to differing peak amplitudes homologous positions do not positionally align between samples).

Due to extremely low variation within the study population, only the SNP on chromosome 14 at base 448009 with an A to T mutation was included in the statistical analysis of impact on attention bias.

Discussion

The genotype frequencies for *HTTLPR*, *TPH2* and *MAOA* in this study were very similar to those of other studies (Barr *et al.* 2013; Karere *et al.* 2012; Ferguson *et al.* 2012). *OPRM1* genotype frequencies matched those of some (Barr *et al.* 2008; Ferguson *et al.* 2012) but not others (Higham *et al*. 2011; Miller *et al.* 2004). Results in this study were replicated several times in order to ensure correct interpretation of each individual's genotype. Further, the sample size of this study was relatively large, compared to a wide range in published studies (Higham *et al.* 2011; Miller *et al.* 2004; Karere *et al.* 2012; Ferguson *et al.* 2012; Watson, Ghodasra & Platt 2009). Although, for a study on non-human primates, the sample size was relatively large, it is by far not the largest available on rhesus macaques (Brent *et al.* 2013; Barr *et al.* 2004b; Watson *et al.* 2015; Spinelli *et al.* 2012; Sullivan, Mendoza & Capitanio 2011). Association studies of humans typically study hundreds of samples in order to achieve reliable results and to be able to detect whether genotype has an impact on observed phenotypes (Caspi *et al.* 2003). However, within the primate community, it is typically not possible to study hundreds of individuals in behavioural or cognitive tests, even when genotyping is possible.

As several females in this study were closely related, genotype frequencies here were not investigated for their distribution in regards to Hardy-Weinberg equilibrium.

Attempts to follow published methodologies often did not yield clear results. Even modification of methods following personal communication with researchers did not improve results (Kinnally, *pers.comm*.; Robinson, *pers.comm*.; Newman, *pers.comm*.). Thus, many published methodologies are deemed not consistent and reliable. It is not entirely clear what cause the problems. All amplification protocols were followed precisely and carried out several times. When this yielded no results, primers were used in different combinations and PCR protocols were changed to trial other temperatures. Further, DNA of animals was extracted at different occasions and other genes were readily analysed. This indicates that there was no issue with the extracted DNA itself. Considering cost and time efforts, methodologies for PCRs using simple agarose or NuSeive gels should be expected to be reliable and easy to repeat. *TPH2* and *OPRM1* were the only two loci for which results were readily analysed and interpretable. However, *MAOA* and *HTTLPR* proved to be notoriously difficult to analyse without replicate amplification and scoring and bands were difficult to score. It also proved

difficult to reliably and consistently obtain PCR products for *DAT1*. Known problems with *HTTLPR* include the fact that this promoter region is difficult to genotype, where the l-allele is sometimes hard to detect (Todkar *et al.* 2013). It has been suggested that conventional PCR product gel electrophoresis is not as good as other, high-throughput methods (capillary electrophoresis) to genotype macaques for the *HTTLPR* and *MAOA* genotype (Karere *et al.* 2012). However, those methods are expensive and were not possible for this study.

Discovery of the novel SNPs in the dopamine receptor *D4*, add to our knowledge of variation within the macaque genome (Gibbs *et al.* 2007; Zimin *et al.* 2014). Future studies could assess the activity of those genotypes *in vitro* in order to be able to suggest possible effects on behaviour. Further, if similar SNPs exist in the human genome, these genotypes could give way to further analysis and increase our knowledge of gene x environment interaction.

Chapter 3. Effect of genotype on attention bias for social stimuli (Experiment 1)

This chapter gives a brief introduction about the possible impact of genotype on attention bias in rhesus macaques. It further describes the methods for the attention bias testing using conspecific male faces. I describe the apparatus used, the procedure for collection of attention bias data, and the statistical analysis. I then present the results in regards of genotype impact on attention bias. Further, the results section includes the results from behavioural observations and genotype impact on behaviour following the attention bias tests. The findings of this experiment are discussed and interpreted in the discussion.

Introduction

In 2007, Beevers *et al.* showed that, in a small sample of 27 psychiatric inpatients, the *HTTLPR* s-allele was connected to a bias towards anxiety-related words. Support for this was found in a study of a healthy population of 111 people, where a strong positive bias for positive material and avoidance of negative material was associated with ll-homozygousity of *HTTLPR* (Fox, Ridgewell & Ashwin 2009). These were the only studies examining attention bias in relation to gene variation.

Anxiety is a heritable trait in rhesus monkeys (Williamson *et al.* 2003) and genetic factors underlying aggression are likely to impact fitness (Brent 2013). It is not unlikely that genetic factors underlying anxiety and attention impact on an individual's fitness, too.

A study by Watson and colleagues (Watson, Ghodasra and Platt 2009) showed that macaques carrying the short *HTTLPR* allele showed enhanced aversion of social threats and were less likely to take a gamble after presentation of a photograph of a high status conspecific male face. They suggested that s-allele carriers experience greater anxiety when viewing potential social threats.

Because the genes investigated in this study have been found to be linked to several behaviours in rhesus macaques as discussed in the Introduction, those were investigated for their impact on AB here. Due to the findings of Watson, Ghodasra and Platt (2009), I hypothesized that the *HTTLPR* short allele would lead to greater vigilance for threatening social stimuli. The other hypotheses as stated in Aim 2, were based on the findings of their impact on other behaviours. I hypothesized that the TPH2 l-allele, the MAOA low-

activity allele and the OPRM1 G-allele would be linked to greater vigilance for threatening stimuli.

This chapter investigated the impact of genotype of the previously mentioned genes on attention bias towards a set of conspecific male stimuli. Several other factors were investigated for their effect on AB performance as well. Those were the age of the tested individual due to the close connection between age and impact of genotype in other studies and the potential for the developmental state a female would be in; previous exposure to AB testing due to overall habituation to being presented with the attention bias paradigm; the side on which each stimulus was presented to the animal due to previous findings of laterality effects in humans and macaques; the nursing status of the tested female (whether she had no baby, a dependent baby or a more independent infant) due to the additional responsibility adding possible 'pressure' to a female to be vigilant for possible threats; the group in which the female lives as there are differences to the amount of individuals in a group as well as differing levels of aggression between individuals; and the number of the current presentation (hence being one for the first presentation, two for the second, three for the third and so on) again, to control for habituation to seeing a specific type of stimulus repeatedly. Those factors were chosen in order to investigate for possible habituation to the stimuli (number of current presentation and previous exposure), social factors such as group and indirect factors which could have an impact on AB such as nursing status.

Materials and Methods

3.1 Animals and Housing

Twenty-eight of the twenty-nine female rhesus macaques, *Macaca mulatta*, for whom genetic data were available, took part in this attention bias study. Monkeys aged between 2.5-15.7 years at the beginning of testing (mean (±SE) = 9.95(±9.01)) and all animals had been socially reared. They were housed in social breeding groups of 6 adult individuals on average with their their offspring at the Centre for Macaques, Medical Research Council (CFM- MRC) in Porton, Wiltshire, England. Harems consisted of a dominant male and a number of females and their infants. Individuals were housed in indoor enclosures that consisted of a cage room and a free roaming room (Fig. 3.1).

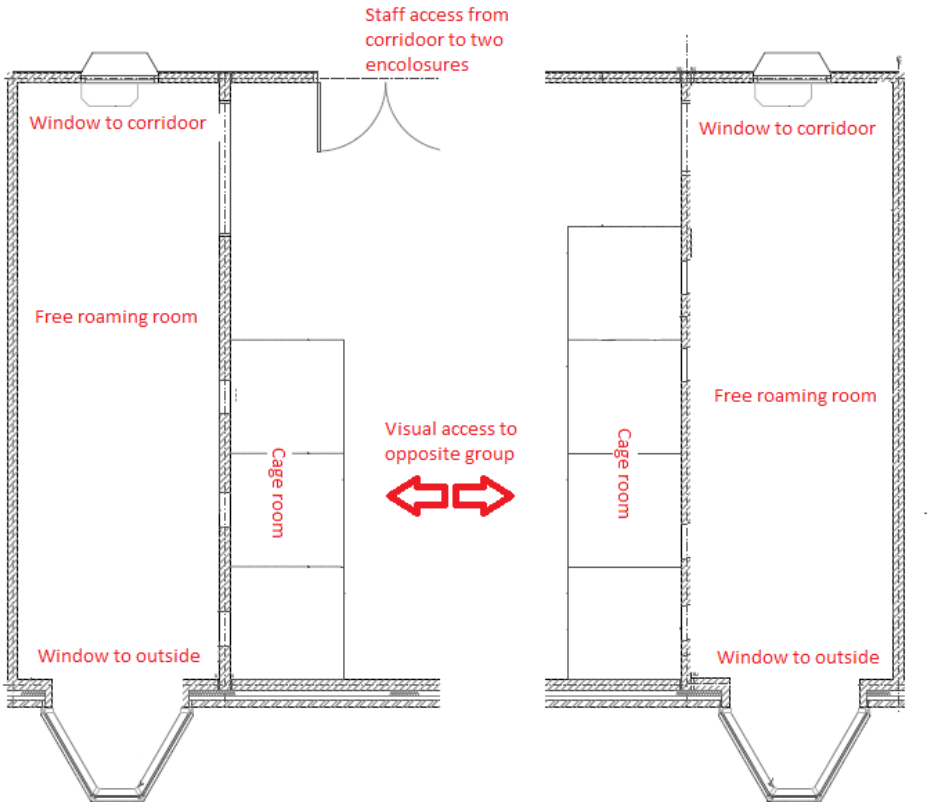

Figure 3.1
Plan of the enclosures. A door from the corridor allowed access to a room containing two cage rooms housing two separate breeding groups of rhesus macaques, *Macaca mulatta*, with visual access to each other. Cage rooms were connected to the free roaming room via hatches in the wall. The free roaming rooms were fitted with windows towards the outside as well as the corridor, from where animals were observed in the free roaming room. Testing took place in the cage room. Figure adapted and used with permission of the Centre For Macaques.

Animals had been previously trained to station within the caging area (Thatcher 2015) using positive reinforcement (Laule, Bloomsmith & Schapiro 2003) and clicker training

(Pryor 1999). All animals except infants were station trained. Animals took part in the attention bias test voluntarily and were free to leave the testing area at any given time. Only females that were well trained to station were tested. Habituation to researchers carrying out the experiment took place over the course of four weeks in the form of hand-feeding and testing commenced once animals were fully habituated to any person present during testing. An animal was declared fully habituated once it comfortably took food from the hand of the researcher and did not display behavioural signs of fear such as fleeing or fear grins. A station was one specific object for each animal (Fig. 3.2) which she was trained to sit by- and, ideally, hold on to during testing (Thatcher 2015). Before attention bias trials started, animals were exposed to the apparatus on approximately five (±1) occasions in order to reduce the novelty of it before testing commenced. A session of attention bias testing for all individuals in the tested group could take between 30 minutes to one hour.

Figure 3.2
Example set of station tools. Each animal was trained to sit by and hold on to a unique station. Photograph taken by H. Thatcher and I.Szott.

3.2 Apparatus

The apparatus (Fig. 3.3) consisted of a wooden pole to which a horizontal part could be attached at two heights. It was custom-made to be at heights that were eye-level to monkeys in the cage rooms depending on the level they sat on. The horizontal part had a simple sliding mechanism operated manually from behind to reveal images simultaneously. There were two gaps at the top through which images could be removed and inserted. The camera which recorded the tested individual was positioned centrally between the two images and, if the horizontal part was brought higher up, was also brought higher up. When the horizontal part was attached at a higher level the tripod on

which the camera stood was placed onto an elevated platform (depending on availability of those in the cage room) or the camera was taped to the pole right beneath it.

Figure 3.3
Apparatus used for presentation of the stimuli and the camera positioned between the stimuli. Here images were a random pair of fruit stimuli. Photograph taken by I.Szott.

3.3 Stimuli

Stimulus pairs were allocated to the animals in a random order. Stimulus pairs for Experiment 1 included an unknown, conspecific male's face with closed eyes (presumed to be neutral) vs. the same male with an aggressive facial expression (presumed to be aggressive, Fig. 3.4). Appendix 4 shows all stimuli pairs. The stimuli with closed eyes were photoshopped, where skin from another part of the face was shopped over the eyes.

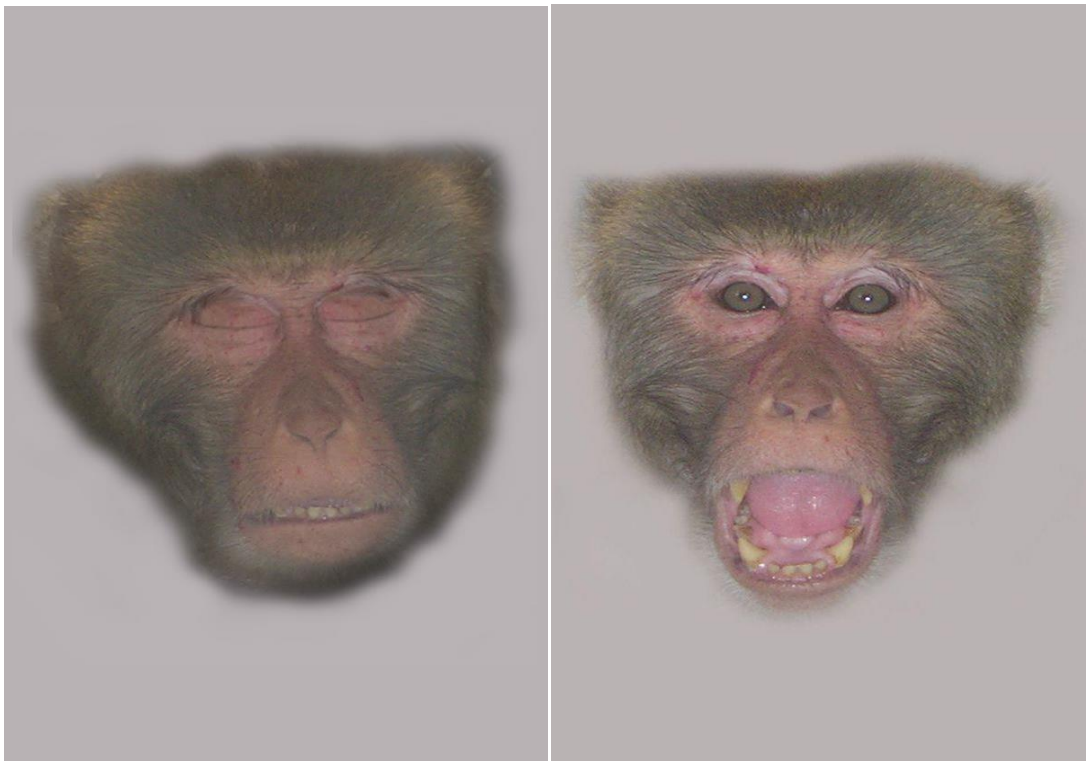

Figure 3.4
Example stimuli pair of an unknown, conspecific male rhesus macaque, *Macaca mulatta*, with an eyes closed neutral facial expression vs. an aggressive facial expression. All stimuli faced inwards. Photographs courtesy of E.Bethell and used with her permission.

Females received four stimulus presentations of unknown conspecific faces over a period of three months. Animals took part in another study (Thatcher 2015) during that time where they received stimuli presentations of unknown male conspecific faces.

Each individual was allocated a pseudorandomised order of presentations of a set of stimuli pairs. In order to avoid habituation to specific stimuli no animal was presented with the same set of stimuli twice. Following the presentation of above mentioned stimuli, females were presented with pairs of images of fruit and vegetables (Fig. 3.5) as a positive stimulus (Thatcher 2015), to avoid the monkeys perceiving the attention bias testing as negative *per se.*

Figure 3.5
Example pairs of vegetable stimuli used as positive stimuli for the attention bias testing of rhesus macaques, *Macaca mulatta*. Photograph taken by H.Thatcher and I.Szott.

3.4 Procedure

Stimuli presentation

Testing took place in the cage room. Two researchers were present during testing, one who loaded the stimuli into the apparatus and one who opened and closed the slides. In order to reduce potential unintended cueing effects, the researcher operating the slides was blind to the set of stimuli that had been loaded. The apparatus was set up in front of a stationed individual to be tested. Following apparatus set-up an animal was encouraged to focus its gaze centrally between the two frames by tapping against the apparatus or holding a piece of food up which was stored in the researchers pocket after the animal oriented its gaze towards the stimuli. Once gaze was directed towards the stimuli, pairs of stimuli were presented. A camera, fixed centrally between the stimuli, recorded the animals head in full view during the trial to capture its gaze. Each stimulus pair was presented for approximately three seconds as counted by the researcher operating the slides. Once the stimuli were presented, the animal was given a food reward to reinforce their taking part in the trial.

Further, as the researcher was standing behind the apparatus to open and close the slides she was visible to the animals. Therefore, the researcher fixed her gaze downwards towards the floor so as not to influence the animal‘s direction of gaze towards the stimuli. During testing, the whole social group was present and stationed. Animals were stationed

in a way to avoid other individuals seeing the stimuli whilst they were not being recorded for attention bias and the order of individuals tested in a group was random and dependent on willingness of individuals to station and take part. If an animal left its station at the moment the stimuli were presented, the trial was aborted, the animal stationed again and the trial then repeated. If an animal that was not being tested left its station during a trial, it was only asked back to its station once the trial had finished. Researchers aimed to not feed any other individuals during the three seconds of a trial so the tested individual would not be distracted.

Each individual was presented with an even number of presentations of both stimuli on each side. The order of presentation of those stimuli pairs as well as the side was allocated in a randomised order and researchers did not know which stimulus was presented in either frame during any particular trial.

Data were collected over the course of three months and each individual had roughly the same amount of days in between each testing session. Animals were tested once a day on two days every other week in order to not interfere with general husbandry and management procedures at the facility. During the week they were tested, each animal was tested once at the beginning of the week (Monday or Tuesday) and once at the end of the week (Thursday or Friday).

Behavioural sampling

No behavioural sampling was carried out previous to testing; hence this study did not investigate whether interactions that took part before testing started (such as aggressive encounters) had an impact on the internal state of the animal. Once the session had been completed and all individuals had been presented with the stimuli, each tested individual‘s behaviour was observed for five minutes using *ad lib* sampling (Altmann 1974) on the JWatcher (Jwatcher.ucla.edu 2014) application following a previously developed and refined ethogram (Table 3.1). Behaviour sampling was carried out through the window between the corridor and free ranging room (Fig. 3.1), avoiding direct eye contact or staring at the observed animal. If an animal went into the cage room of the enclosure and could not be seen by the observer, it was classed as out of sight. If the animal was out of sight for more than two and a half minutes, the observation was aborted and re-done once the animal was back in sight. Due to some animals choosing to stay in the cage room for a prolonged period of time, those were observed through the

door between the cage room and the corridor and a note was made highlighting that the animal was observed in the cage room.

Table 3.1. Behavioural ethogram used for *ad lib* sampling of female rhesus macaques, *Macaca Mulatta*, after attention bias testing.

Code	Behaviour	Description
0	Out of sight	Animal is either in the cage room or behind an object within the free ranging room and not visible to the observer
C	Aggressive	Animal actively attacks a conspecific by chasing, biting or hitting it.
F	Affiliative	Animal is sitting in contact with another conspecific, lip smacks at a conspecific that is in close proximity but is not grooming or being groomed.
U	Submissive	Animal moves out the way of a conspecific, ducks away from it or screams in a high pitched manner. Animal can also present its hindquarters in a non-sexual context (not followed by mating)
a	Lip smack with fear grin	Animal opens and closes its lips repeatedly without showing its teeth and retracts it's lips so that clenched teeth are exposed
b	Interaction with baby	Animal interacts with any baby of the social group. Interactions could be grooming, playing, carrying the infant or taking food from it
d	Displace	Animal approaches a conspecific and the approached animal leaves its current occupied space
e	Fleeing	Animal rapidly runs away from a conspecific
f	Foraging	Animal is eating food, holding it in its hand or searching through the wood chippings for seeds and other food
g	Allogrooming	Animal is picking through the fur or over the skin of another individual using it's hand or mouth
h	Being groomed	Animal's fur or skin is picked through by a conspecific
i	Self-directed	Any behaviour, such as grooming the own fur, scratching or picking scabs directed at the own body
k	Body shake	Animal rapidly moves its whole body, usually starting with a quick shaking of the head followed by the whole body.
l	Locomotion	Animal moves through the enclosure but not fleeing from- or chasing another individual
m	Lip smack	Animal opens and closes its lips repeatedly without showing its teeth, making a smacking sound on occasion
n	Fear grin	Animal bares its teeth in a grimace and protracts it's lips
o	Object	Animal interacts with something in the enclosure such as ropes, a mirror or plastic toys but not conspecifics or humans

p	Displaced	Animal is approached by a conspecific and leaves its current occupied spot
r	Resting	Animal is sitting or lying down with its eyes closed for a prolonged amount of time
s	Stationary	Animal is sitting or lying down in one spot and does not actively scan its surroundings but has its eyes opened and
t	Threat	Animal rapidly and aggressively lunges its body towards a conspecific or person sometimes whilst slapping the ground with one hand or whilst having its mouth opened without seeing the teeth. The animal does however not make physical contact
v	Vigilance	Animal actively observes its surroundings, looking around and moving its eyes or head scanning the surroundings
x	Sexual behaviour	Animal presents or is presented the hindquarters. Alignment of the males hindquarters with the haunches of the females
y	Yawn	Animal opens its mouth wide open
z	Stereotypic behaviour	Animal performs a certain bout of behaviour repeatedly, such as pacing up and down, walking up to a wall and pushing itself off it repeatedly, or engaging in bar biting

3.5 Video coding

Recording trials on video allows for more precise and reliable scoring of data compared to visual assessment of an individual's gaze, as well as allowing for assessments of inter-rater reliability (Winters, Dubuc & Higham 2015). Videos of the attention bias trials were blind-coded for gaze towards the stimuli. Video coding was done using a gaze ethogram (Table 3.2) in JWatcher. If an animal looked towards the right of the computer screen from the coders' perspective, it was assumed to look at the stimulus presented to its left from the animals perspective and *vice versa*. The video was cut to start just at the moment the slides were opened and the female could see the stimuli and to end at exactly three seconds from this. The gaze was coded for those three seconds frame-by-frame. For statistical analysis the overall duration in milliseconds a female spent looking at either stimulus within those three seconds was considered. For this study, about one third of trials were coded by two observers at the same time whilst another third was double coded and rated for inter-observer reliability, achieving a Kappa-coefficient score of 84%. Once coded, the first three seconds were used for analysis in order to analyse the pre-conscious and initial reaction of an individual to the presented stimuli (see introduction). Because stimuli were presented for approximately three seconds as

counted by the researcher operating the slides, this use of the exact first three seconds allowed for comparison between all females.

Table 3.2. Gaze ethogram used for blind-coding of video footage of female rhesus macaques, *Macaca mulatta*, for gaze towards stimuli that were located towards the right and the left of the camera.

Code	Behaviour	Description
0	First look right	First time the animal looked at the stimulus on the right hand side of the screen
1	First look left	First time the animal looked at the stimulus on the left hand side of the screen
A	Away	The animal looks at a point that cannot be classed as any of the other 'away' categories
B	Baby	The animal looks at- and sometimes huddles its baby
C	Central	The animal looks at a point between the two stimuli
D	Away down	The animal looks down towards the apparatus but not at the stimuli
I	Away up right	The animal looks to the top right corner of the room, but not at the stimuli
J	Away up extreme	The animal looks centrally above itself, turning its head up so the chin is facing up
K	Away left	The animal looks away to the left hand side of the room
L	Look left	The animal looks at the stimulus in the left side of the screen
N	Away down extreme	The animal looks down towards the floor and turning its head down so the top of the head is facing the camera
O	Out of view	The eyes cannot be seen and the direction of gaze is not obvious
R	Look right	The animal looks at the stimulus to the right side of the screen
T	Away right	The animal looks away to the right hand side of the room
U	Away up central	The animal looks upwards, but not at the apparatus or stimuli
Y	Away up left	The animal looks to the top left corner of the room, but not at the stimuli

After videos were coded, they were matched with known location (side) of the negative and neutral stimulus in order to find the overall time spent looking at each stimulus

during each trial. Only the time an individual spent looking directly at the stimuli was considered for analysis.

3.6 Statistical analysis of influence of genotype and other factors on AB

Associations between attention bias and genotypes either at individual loci or at the multi-locus level were examined in the *R Statistical software* package (version 3.2.0, R Core Team 2015), using generalised linear mixed effects models (GLMM) and the lme4 (Bates *et al.* 2015) and MuMIn (Barton 2015) packages. Genotypes were classed into two categories (Table 3.3) based on whether they were low- or high expressing alleles. Low expressing alleles, such as the HTTLPR s-alelle lead to less serotonin in the brain, whilst high expressing alleles lead to an increased amount (Table 3.3). I hypothesized that the low expressing alleles would lead to increased vigilance for threat. For the novel *DRD4* SNPs, the A-allele was more common and hence labelled the high-expressing genotype, in accordance of classification of other genotypes used in this study. However, it is important to note that no *in vitro* studies were carried out as to whether this allele was actually linked to higher *in vitro* expression of dopamine. The classification of 'opportunistic' and 'conservative' for the *MAOA* genotypes is linked to the fact that it is X-linked (see introduction). When interpreting the genotype classes 'opportunistically' it was assumed that when individuals carried only one 7-repeat allele, it was not active. When classed 'conservatively' the presence of any 7-repeat allele was treated as if it were active. Hence, it was a matter of 'hoping for the best' (opportunistic) *vs.* 'expecting the worst' (conservative). Because previous studies found that the combination of the *HTTLPR* and *TPH2* genotype had an impact on behaviour, this combination was also included and investigated in this study.

Table 3.3. Categorisation of genotypes. 'l' long allele, 's' short allele. C, G, A and T nucleotide at the studied position. *MAOA* 5, 6 and 7 repeats within the studied region. Categorisation was based on findings in the existing literature (see introduction). *HTTLPR* and *TPH2* were studied in combination due to findings in previous studies (Brent *et al.* 2013). Genotypes which are hypothesized to lead to greater vigilance for threat are the *HTTLPR*, *TPH2*,*OPRM1* and *MAOA* low expressing genotypes based on findings in previous studies.

Genotype	High-expressing (leading to increased amounts of circulating neurotransmitters in the brain)	Low-expressing (leading to decreased amounts of circulating neurotransmitters in the brain)	Link to behaviour in rhesus macaques, *Macaca mulatta*
HTTLPR	ll: ll-homozygous	sl, ss: s-allele carriers	s-allele: increased anxiety-related behaviours, environmental

			exploration, self-directed behaviour following stress, delayed early neurobiological development, impaired serotonergic function, excessive aggression, HPA reactivity, alcohol consumption, superior cognitive performance (see Chapter 1)
TPH2	ss: ss-homozygous	sl, ll: l-allele carriers	l-allele: self-injurious behaviours, altered HPA axis function, decreased vigilance in social context (see Chapter 1)
OPRM1	CC: CC-homozygous	CG, GG: G-allele carriers	G-allele: increased aggressive threat behaviours, greater levels of alcohol consumption, higher restrainment rates of infants by mothers, increased opiate reward in response to affiliation, higher baseline attachment behaviours (see Chapter 1)
MAOA	Opportunistic: 55, 56, 66, 57, 67: non 77-homozygous Conservative: 55, 56, 66: no 7-allele carriers	Opportunistic: 77: 77-homozygous Conservative: 57, 67, 77 : 7-allele carriers	7-repeat allele: impulsive and aggressive behaviour, increased stress reactivity (see Chapter 1)
DRD4	AA: AA-homozygous	AT, TT: T-allele carriers	*DRD4* length polymorphism: exploration and risk-taking, physical aggression towards conspecific (note: this is a different polymorphism than the one identified in this study; see Chapter 1)
HTTLPR and *TPH2 (HTT-TPH)*	*HTTLPR* ll + *TPH2* ss: Combination 1 carriers	*HTTLPR* sl + *TPH2* sl *HTTLPR* sl + *TPH2* ll, *HTTLPR* ss + *TPH2* sl, *HTTLPR* ss + *TPH2* ss, *HTTLPR* ll + *TPH2* ll: Combination 2 carriers *HTTLPR* ss + *TPH2* ll: Combination 3 carriers (not present in this study)	Combination 2 or 3: fewer friends and allies (see Chapter 1)

In order to investigate whether genotype had an impact on attention bias, it was necessary to identify and control for all recorded explanatory variables. The following factors were included in the maximum model: Monkey identity, matriline, social group (group 1-7), age, previous exposure (whether the individual took part in attention bias

studies previous to the current ones both this study and Thatcher 2015), nursing status (no baby, nursing a dependent baby that stays within close proximity of its mother or a more independent infant that spends more time away from its mother), side of stimulus presentation (negative stimulus presented to the monkeys left or right) and the number of stimuli presentations. Stimuli presentation controlled for the number of times a female had been presented with unknown conspecific stimuli overall at the current point of testing, in order to control for a possible habituation effect to exposure to stimuli overall. This included all conspecific stimuli: from this study as well as the study carried out simultaneously (Thatcher 2015). Hence, the first time an individual was tested it was presentation 1, whilst the last time she was tested it was presentation 12.

The cbind command was used to form the response variable (AB). Each trial consisted of three seconds. Within those three seconds there were two variables: overall time spent looking at the aggressive conspecific face and the overall time spent looking at the presumed to be neutral conspecific face. Those two variables were used to create the new response variable. The so called *binomial denominator, n*, is the total sample of the two respective variables; hence the overall duration in milliseconds, spent looking at the aggressive and neutral stimulus in this specific trial.

time spent looking at aggressive = binomial denominator – time spent looking at neutral

The following was the code used:

AB<-cbind (Aggressive, Neutral)

Further, this command controlled for variation between individuals, where some might have spent the full three seconds looking at the stimuli, whilst others might have looked away and only glanced at both stimuli for a fraction of the three seconds.

Preliminary analysis for collinearity was carried out by using the cor(dat) command (Becker, Chambers & Wilks 1988). Previous exposure correlated with matriline (r=-0.31) as well as age (r=0.49, $p \leq 0.001$ for all tests). Previous exposure was not included in the analysis.

Due to the nature of the samples utilised in this genetic study which unavoidably included related individuals, it was necessary that monkey identity was nested within its matriline to partially control for relatedness. The number of presentations an individual had at the

point of testing was included as a random factor and matriline and monkey ID were included as nested random factors. Age of the female in months (AgeMos), group in which the female lived (Group), nursing status as no infant/baby=1, nursing baby=2, independent infant=3 (NursStat) and side as left=0 and right=1 (Side) on which the aggressive stimulus was presented to the animal were also included as fixed factors in the maximum model:

glmer(AB ~ AgeMos + Group + NursStat + Side + (1|Presentation) + (1|Matriline/MonkeyID), family=binomial)

From this maximum model a list of 30 candidate models with binomial error distribution were created (Appendix 5). Using the Akaike Information Criterion (AICc) scores (Crawley 2007) the model with the lowest value indicated the best fit model. All models within 2 AICc points of the best fit model were kept as the final set of models (Crawley 2007).

To test for an effect of fixed factors from the best-fit models in combination with genotypes on attention bias (AB), test models were created. Those test models (with random factors held constant) included each fixed factor from the previous best-fit models in combination with the genotype factors. The following maximum model was designed:

glmer(AB ~ Side + NursStat + HTTLPR + TPH2 + MAOA + OPRM1 + DRD4 + (1|Presentation) + (1|Matriline/MonkeyID), family=binomial)

To investigate whether *HTTLPR* and *TPH2* in combination had a larger effect (Brent *et al.* 2013), the best fit model from the above list was compared to one that included the new variable *HTT-TPH* (Table 3.3). Combination 3 was not present within the studied population, so combinations 1 and 2 were compared. However, AIC scores showed that in combination, *HTTLPR* and *TPH2* did not have greater power at explaining AB data and this model was therefore not considered further.

When looking at a best fit model, there is a potential for variables not being significant (Crawley 2007). Weighted regression analysis was then used to select the best model by carrying out a deletion test. To do this, a simpler model is created, missing the least significant variable of the previous model. A Chi-squared test by running an ANOVA between the two models was carried out (Crawley 2007). As the response variables were

binomial data, a binomial error structure was used. A *p*-level of $p \leq 0.05$ was used for significance.

In order to plot the attention bias score, the following equation was used

AB= (Aggressive / (Aggressive+Neutral)) – 0.5

By subtracting 0.5, vigilance towards a stimulus was represented by a positive value, whilst a negative value indicated avoidance of a stimulus.

3.7 Statistical analysis of behavioural observations

Differences in behaviour during the behavioural observations were examined in SPSS (version 22). In order to analyse the behavioural observation data, the behaviours from the ethogram (Table 3.2) were classed into five distinct categories (Table 3.4).

Table 3.4. Behavioural categories for statistical analysis and the behaviours they included from the behavioural sampling of female rhesus macaques, *Macaca mulatta*.

Category	Behaviours included
Aggressive approach	Aggressive, Displace, Threat
Affiliative approach	Affiliative, Lip smack, Grooming, Groomed
Fear avoid	Submissive, Displaced, Flee, Fear grin, Lip smack with fear grin
Self-directed and anxiety	Self-directed, Yawn, Stereotypy, Vigilance, Body shake
Maintenance	Interaction with baby, Foraging, Locomotion, Object, Resting, Stationary, Sexual behaviour

Normality distribution of these behavioural categories was assessed (Appendix 6) and the appropriate tests were used. Bonferroni correction for multiple tests (Dunn 1961) led to a significance level of $p \leq 0.005$ and results are presented for significance assigned at the $p \leq 0.05$ level, as well as the Bonferroni corrected value.

In order to test whether there was a difference between the influence of the two genotype classes on each type of behaviours displayed, a Mann-Whitney *U* test (Independent-samples *t* test for self-directed and maintenance) was performed.

3.7 Effect of genotype on attention bias: aggressive *vs* neutral conspecific face

HTTLPR and *MAOA* genotype had a significant impact on attention bias. When *MAOA* was classed with 7-repeat allele carriers as low-expressing and non-carriers as high expressing (conservative), the best fit model included side, nursing status, *HTTLPR* and *MAOA* to influence AB data as separate factors. A new list of models investigated whether any of those factors in interaction explained the AB data better than just each factor alone. The best fit model from this model selection showed an interaction between side and nursing status in addition to *HTTLPR* and *MAOA* (Table 3.5). This model met the assumptions of normality (Appendix 7).

Table 3.5. AIC_c - ranked candidate models showing the relative importance of fixed effects (*HTTLPR*, *MAOA*, side, nursing status) and random effects (presentation, monkey ID and matriline) in explaining attention bias of female rhesus macaques, *Macaca mulatta*.

Fixed effects	Random effects (/nested)	d.f.	Log likelihood	AIC	delta
***HTTLPR*, *MAOA*, Side x Nursing status**	(Matriline/ MonkeyID), Presentation	11	-13899.16	27823.0	0.00
***HTTLPR*, Side x Nursing status**	(Matriline/ MonkeyID), Presentation	10	-13901.40	27825.0	2.03

3.8 Weighted regression analysis

To ascertain that this model did in fact explain AB data better than any other model, further model selection was carried out. The current best fit model was compared to a second model which did not include the previous least significant factor, in this case *MAOA* (p=0.022). An *ANOVA* revealed that removing *MAOA* did change the significance of the model explaining the AB results (χ^2=4.8171, p=0.028). Hence, *MAOA* genotype had a significant impact on attention bias.

The final model that best explained an individual's attention bias included the number of presentations the individual had received at that point as a fixed factor (the number of presentations at the current point of testing, in order to control for a possible habituation effect to exposure to stimuli overall), *HTTLPR* (Fig. 3.7), *MAOA* (Fig. 3.8), and an

interaction between the side the negative stimulus was presented on, with the nursing status of the female (Fig. 3.9, Table 3.6). Side and nursing status were both significant by themselves, too (Table 3.6). *HTTLPR* s-allele carriers and non-carriers of the *MAOA* 7-repeat allele were more avoidant of the aggressive stimuli compared to ll-homozygous females and 7-repeat allele carriers respectively. Females that had no baby or a nursing baby were more vigilant for the neutral stimuli overall and showed higher avoidance of aggressive stimuli presented on their left. Females with an older infant were more vigilant for the aggressive stimuli compared to neutral stimuli.

Table 3.6. Factors included in the best fit model and their *z*- and *p*- values for impact on attention bias of female rhesus macaques, *Macaca mulatta*. Significance assigned at $p \leq 0.05$*, $p \leq 0.005$**, $p \leq 0.001$***.

Model	*z*-value	*p*-value
HTTLPR	$z=2.87$	$p=0.00407$**
***MAOA* conservative**	$z=2.29$	$p=0.02177$*
Side x Nursing status	$z=-46.29$	$p<0.001$***
Side	$z=55.12$	$p<0.001$***
Nursing status	$z=4.95$	$p<0.001$***

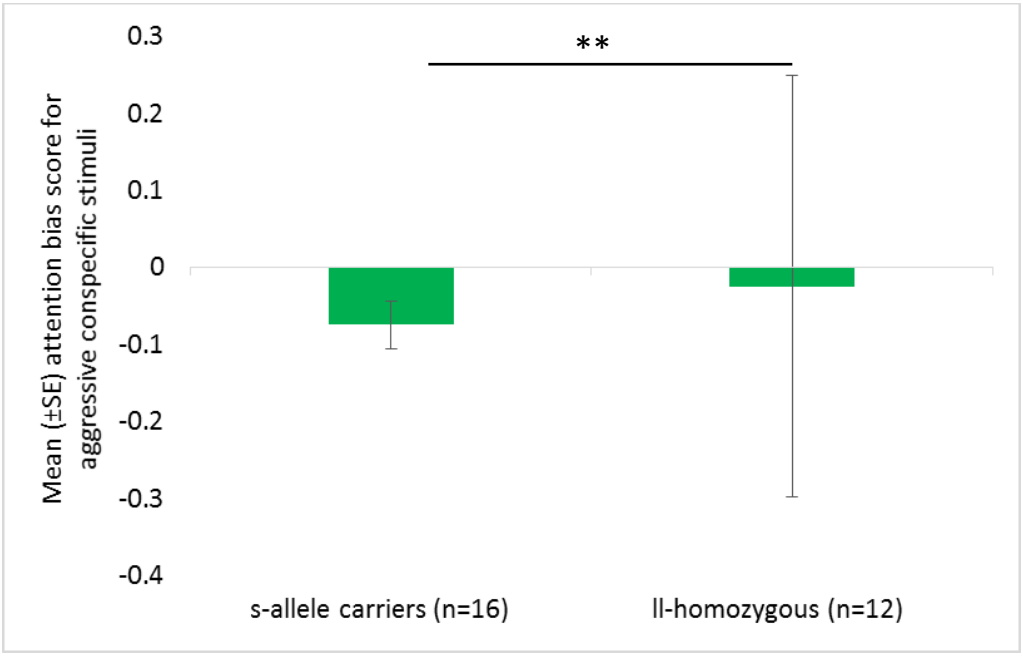

Figure 3.7
Mean (±SE) attention bias score of rhesus macaque, *Macaca mulatta*, females (n=28) for the aggressive (aggressive facial expression) and neutral (eyes closed, neutral facial expression) male conspecific stimuli, depending on their *HTTLPR* genotype (s-allele carriers (n=16), ll-homozygous

(n=12)). Positive values indicate vigilance for- and negative values avoidance of the aggressive stimuli. Significance assigned at p≤0.05*, p≤0.005**.

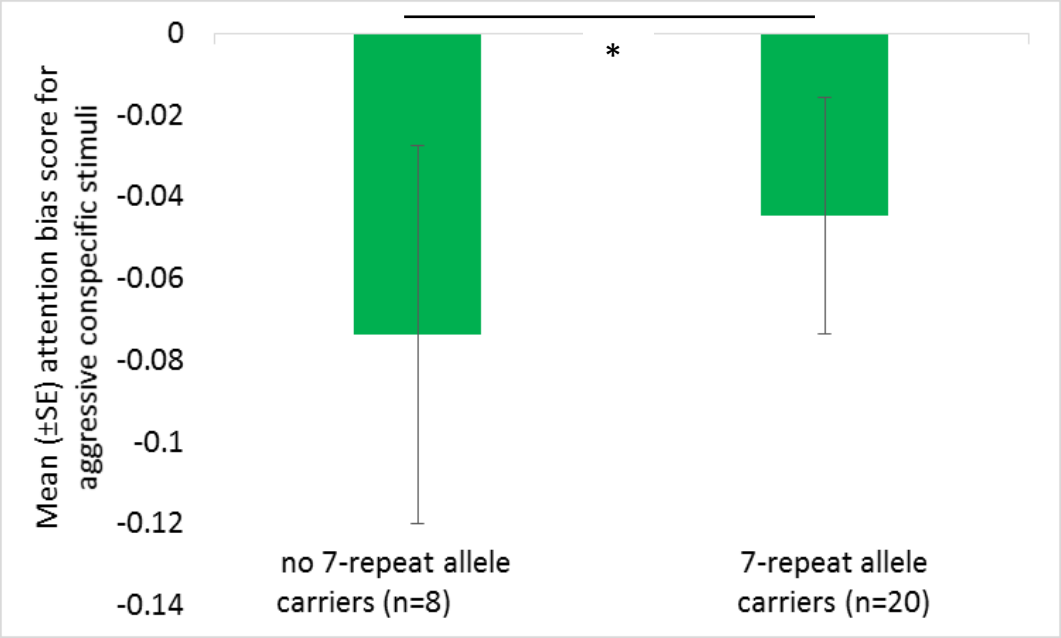

Figure 3.8
Mean (±SE) attention bias score of rhesus macaque, *Macaca mulatta*, females (n=28) for the aggressive (aggressive facial expression) or neutral (eyes closed, neutral facial expression) male conspecific stimuli, depending on their *MAOA* genotype (7-repeat allele carriers (n=20), no 7-repeat allele carriers (n=8)). Positive values indicate vigilance for- and negative values avoidance of the aggressive stimuli. Significance assigned at p≤0.05*, p≤0.005**, P≤0.001***.

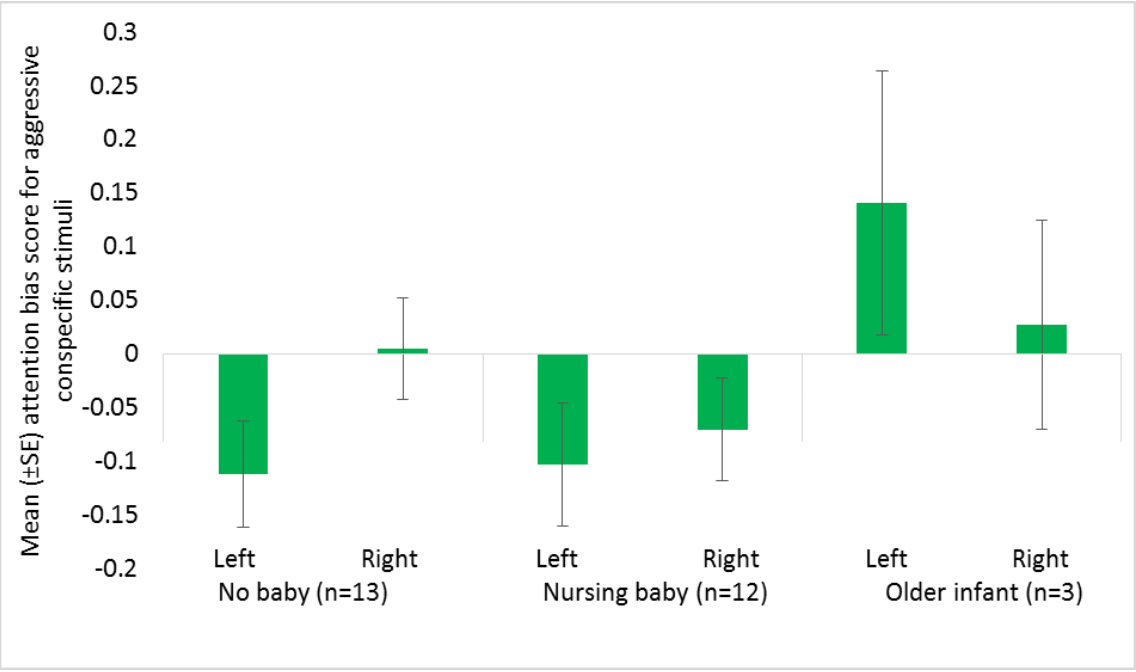

Figure 3.9
Mean (±SE) attention bias score of rhesus macaque, *Macaca mulatta*, females (n=28) for the aggressive (aggressive facial expression) or neutral (eyes closed, neutral facial expression) male conspecific stimuli, depending on their nursing status (no baby (n=13), nursing baby (n=12), older infant (n=3)) and the side on which the negative stimulus was presented to them. Positive values indicate vigilance for- and negative values avoidance of the aggressive stimuli.

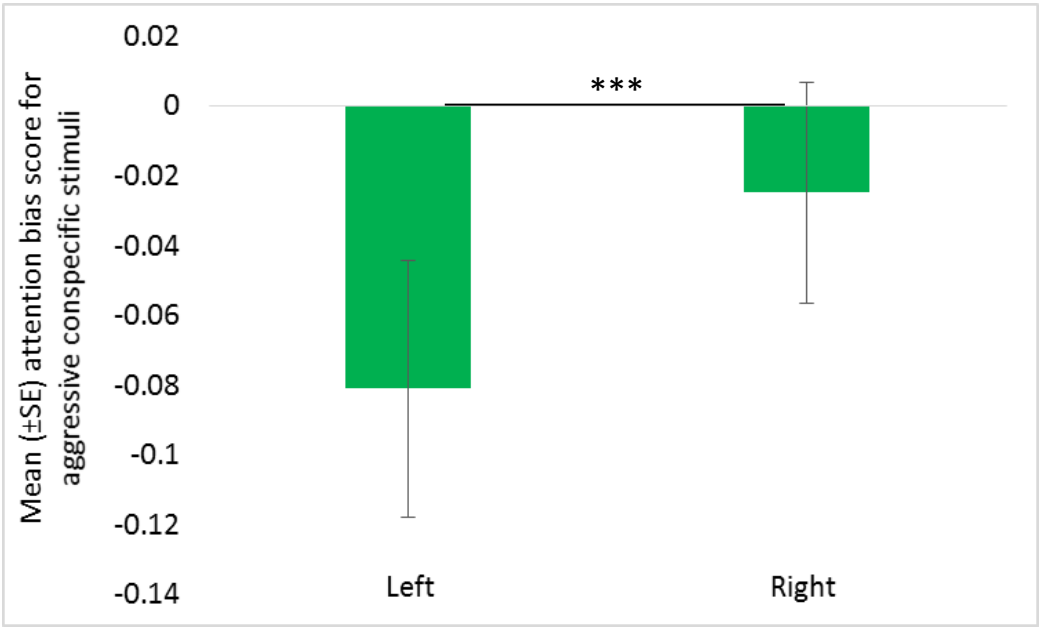

Figure 3.10
Mean (±SE) attention bias score of rhesus macaque, *Macaca mulatta*, females (n=28) for the aggressive (aggressive facial expression) or neutral (eyes closed, neutral facial expression) male conspecific stimuli, depending on the side the aggressive stimulus was presented to them. Positive values indicate vigilance for- and negative values avoidance of the aggressive stimuli. Significance assigned at p≤0.05*, p≤0.005**, P≤0.001***.

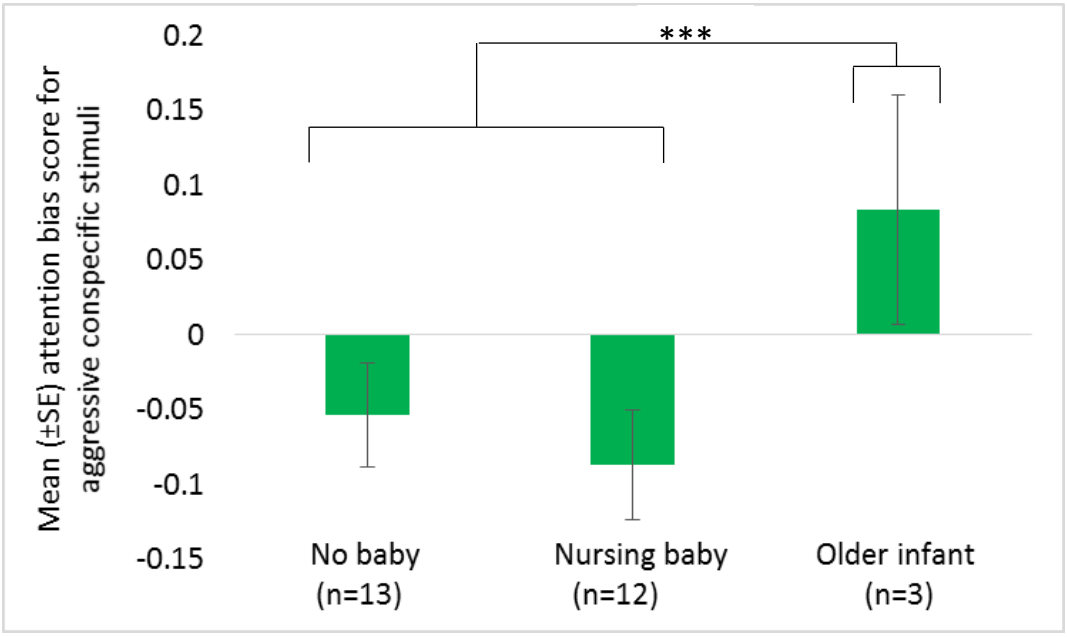

Figure 3.11
Mean (±SE) attention bias score of rhesus macaque, *Macaca mulatta*, females (n=28) for the aggressive (aggressive facial expression) or neutral (eyes closed, neutral facial expression) male conspecific stimuli, depending on their nursing status (no baby (n=13), nursing baby (n=12), older infant (n=3)). Positive values indicate vigilance for- and negative values avoidance of the aggressive stimuli. Significance assigned at p≤0.05*, p≤0.005**, P≤0.001***.

This result was achieved when *MAOA* was classed with all females carrying a 7-allele in one group (conservative interpretation). The number of 77-homozygous females was too

small (n=3) to run analyses with those compared to females carrying only one 7-repeat allele or none.

3.9 Does genotype influence the behaviours displayed?

For aggressive approach there was a weak significant difference between the two *DRD4* genotype classes, where females carrying one or two T-alleles performed significantly more aggressive behaviours compared to females homozygous for the A-allele (Table 3.7, Fig. 3.12). *MAOA*, when interpreted conservatively (see methods), showed significantly more individuals carrying one or two 7-alleles performed affiliative approach behaviours (Fig. 3.13) and significantly less maintenance behaviours (Fig. 3.14) compared to individuals carrying none (Table 3.7). These values did not withstand Bonferroni correction.

There was no significant difference between any of the other genotype classes on aggressive, affiliative or maintenance behaviours displayed (Table 3.7). There was no significant effect of the genotype classes on fear behaviours or self-directed behaviours (Table 3.7).

Table 3.7. *U*- or *t*-values (d.f.) and *p*- values of impact of genotype classes on behavioural categories performed after attention bias testing by female rhesus macaques, *Macaca mulatta,* before Bonferroni correction. Significance assigned at $p \leq 0.05$*.

Behaviour	Genotype	*U*-value/ *t*-value (d.f.)	*p*-value
Aggressive	*HTTLPR*	60	0.088
	TPH2	68	0.566
	MAOA conservative	74	0.784
	OPRM1	26	0.164
	DRD4	45	0.048*
	HTTLPR and *TPH2* combined	15	0.106
Affiliative	*HTTLPR*	82	0.496
	TPH2	67.5	0.520
	MAOA conservative	35	0.021*
	OPRM1	39.5	0.590
	DRD4	82.5	0.885

	HTTLPR and *TPH2* combined	26	0.433
Fear	*HTTLPR*	61.5	0.098
	TPH2	69	0.535
	MAOA conservative	74.5	0.757
	OPRM1	42.5	0.728
	DRD4	83.5	0.923
	HTTLPR and *TPH2* combined	35	0.889
Self-directed	*HTTLPR*	0.376 (26)	0.710
	TPH2	0.486 (26)	0.631
	MAOA conservative	-0.101 (26)	0.921
	OPRM1	-0.201 (26)	0.842
	DRD4	-0.738 (26)	0.467
	HTTLPR and *TPH2* combined	0.589 (26)	0.561
Maintenance	*HTTLPR*	-0.414 (26)	0.682
	TPH2	-0.333 (26)	0.742
	MAOA conservative	2.670 (26)	0.013*****
	OPRM1	0.199 (26)	0.844
	DRD4	0.395 (26)	0.696
	HTTLPR and *TPH2* combined	-0.535 (26)	0.597

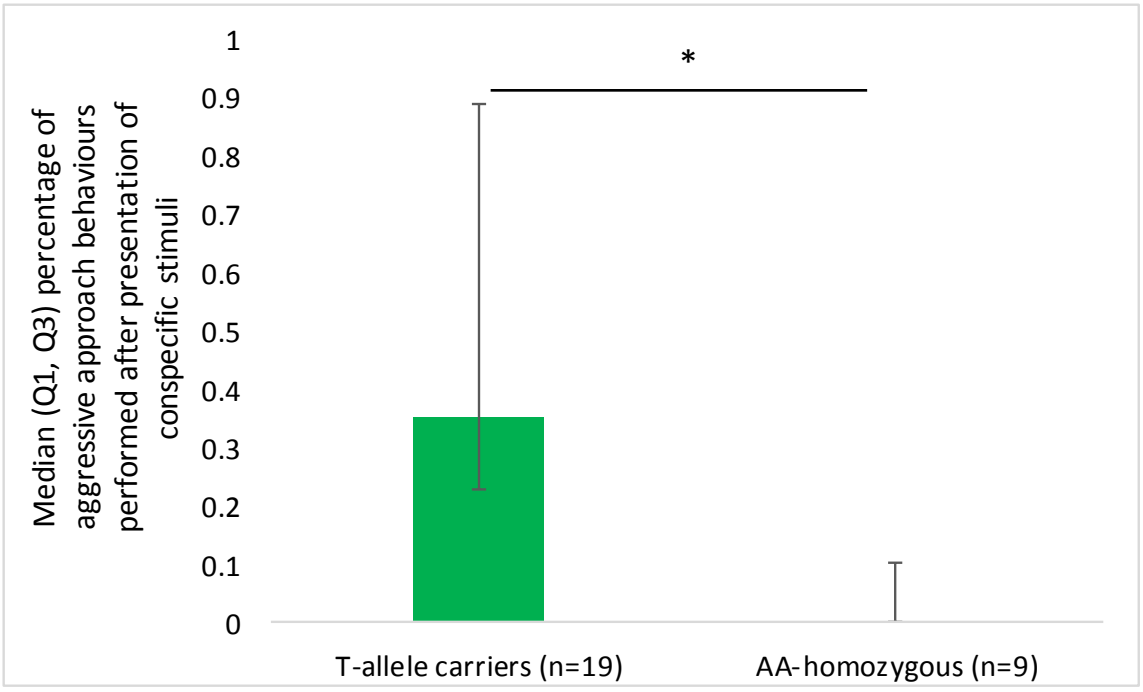

Figure 3.12
Median (Quartile 1 and 3) percentage of aggressive approach behaviours displayed by rhesus macaque, *Macaca mulatta*, females, after presentation of aggressive *vs.* eyes closed conspecific stimuli, depending on their *DRD4* genotype (T-allele carriers (n=19) and non-carriers (n=9)). Significance assigned at p≤0.05*.

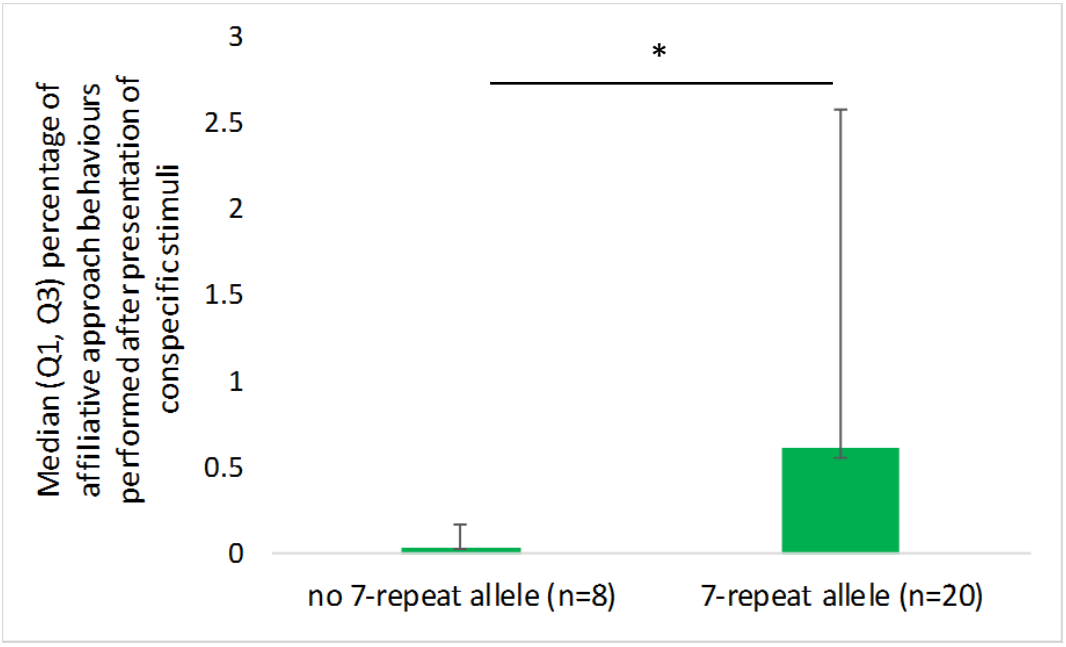

Figure 3.13
Median (Quartile 1 and 3) percentage of affiliative approach behaviours female rhesus macaques, *Macaca mulatta*, displayed after presentation of aggressive *vs.* eyes closed conspecific stimuli, depending on their *MAOA* genotype, when interpreted conservatively (no 7-repeat allele carriers (n=8), 7-repeat allele carriers (n=20)). Significance assigned at p≤0.05*.

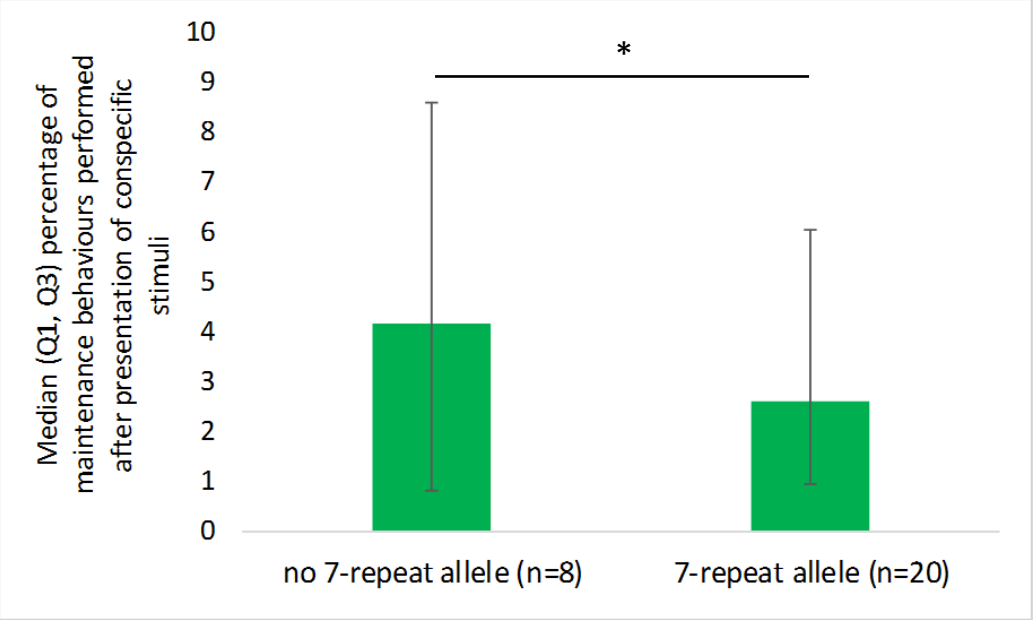

Figure 3.14
Median (Quartile 1 and 3) percentage of maintenance behaviours displayed by rhesus macaque, *Macaca mulatta*, females after presentation of aggressive *vs.* eyes closed conspecific stimuli, depending on their *MAOA* genotype, when interpreted conservatively (no 7-repeat allele carriers (n=8), 7-repeat allele carriers (n=20)). Significance assigned at p≤0.05*.

The non-significant trend of *HTTLPR* genotype classes showed that s-allele carriers performed more aggressive behaviours (Fig.3.15) and less fear behaviours (Fig. 3.16) on average compared to ll-homozygous females (Table 3.7).

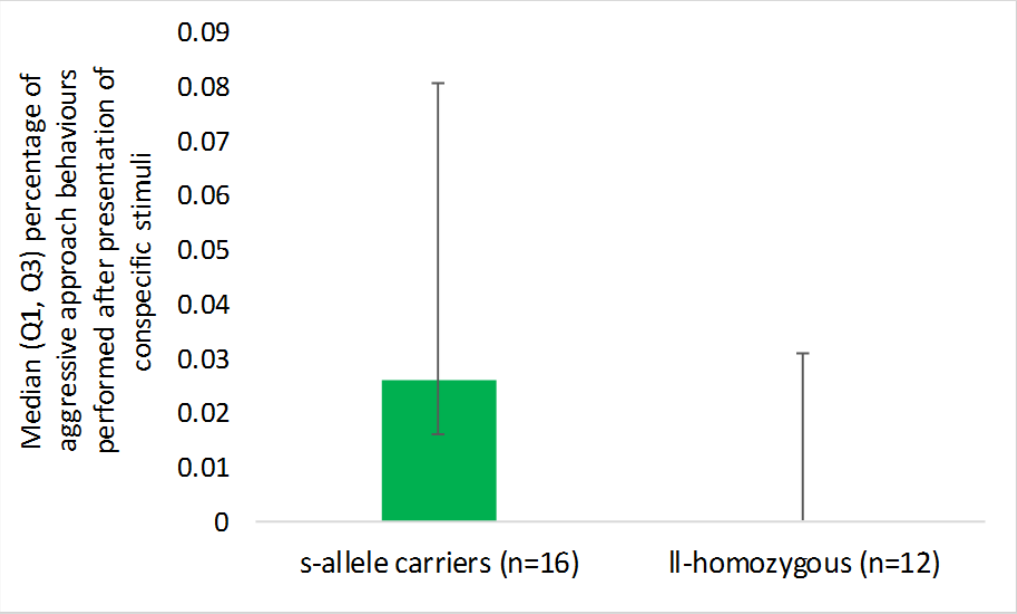

Figure 3.15
Median (Quartile 1 and 3) percentage of aggressive approach behaviours rhesus macaque, *Macaca mulatta*, females displayed after presentation of aggressive *vs.* eyes closed conspecific stimuli, depending on their *HTTLPR* genotype (s-allele carriers (n=16) and non-carriers (n=12)).

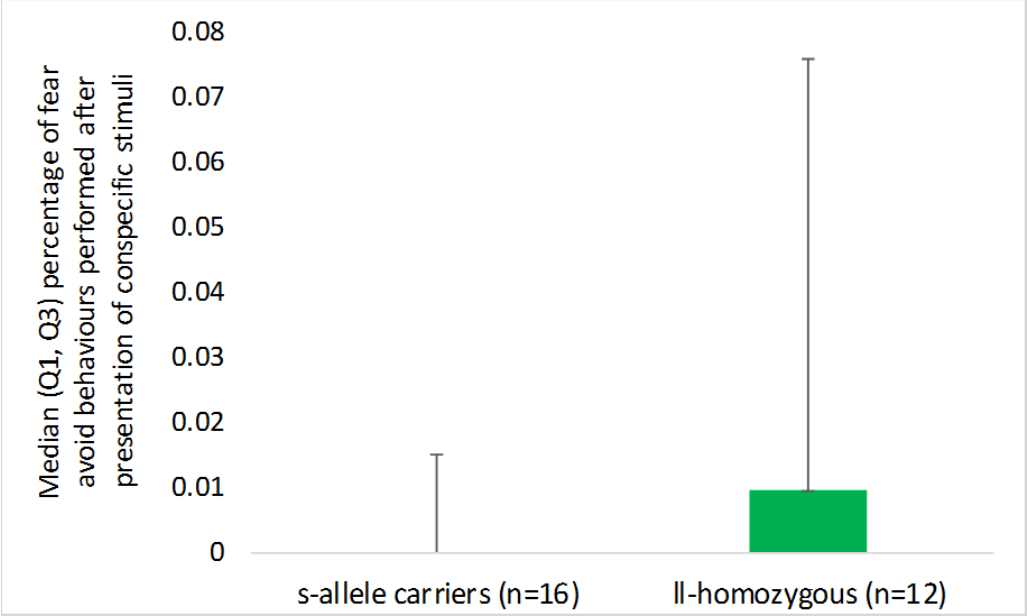

Figure 3.16
Median (Quartile 1 and 3) percentage of fear behaviours female rhesus macaques, *Macaca mulatta*, displayed after presentation of aggressive *vs.* eyes closed conspecific stimuli, depending on their *HTTLPR* genotype (s-allele carriers (n=15) and non-carriers (n=13)).

Discussion

Impact of genotype on attention bias for social stimuli (unknown conspecific)

In this study, *HTTLPR* and *MAOA* genotype have been shown to have an impact on attention bias towards social stimuli (unknown, conspecific faces), where the *HTTLPR* s-allele and the *MAOA* 5- or 6-repeat allele only carriers were more avoidant of aggressive conspecific faces. Additionally, other factors influencing attention bias have been identified. Attention for conspecifics was influenced by an interaction of the nursing status of the tested female, as well as the side on which the aggressive stimulus was presented to her. Further to that, side generally had an impact, where, if the aggressive stimuli were presented on the left hand side, females avoided looking at those at a higher rate whilst if the aggressive stimuli were presented on the right both stimuli received attention. Generally, all animals looked more at the neutral compared to the aggressive stimuli.

The impact of *HTTLPR* on attention bias in general seems to contradict the findings of Beevers *et al.* (2007) and Fox, Ridgewell & Ashwin (2009) on humans. There, ll-homozygous humans avoided negative material and focused on positive material, whilst they did not show such a significant trend in s-allele carriers. However, whilst humans have been found to be more vigilant for negatively valenced stimuli when they carry the s-allele, macaques seemed to be more avoidant, a finding in line with results by Bethell *et al.* (2012a). One result that seems to be consistent across studies (Bethell *et al.* 2012a; Fox Ridgewell & Ashwin 2009) is that individuals focus more on neutral stimuli in general compared to negative stimuli. The results here do align with the findings that macaque s-allele carriers avoid dominant macaque faces (Watson, Ghodasra & Platt 2009). Their study was carried out on a very small sample size (4-8 individuals) of pair housed males with unknown rearing history and the monkeys were on controlled fluid access outside of testing sessions, earning roughly 80% of their total daily fluid ration during experiments (Watson, Ghodasra & Platt 2009). Those adverse testing conditions and small sample sizes could have impaired the validity of the results but this study found support of their findings. Overall, studies on the relationship between macaque's attention and aggressive or dominant conspecific faces show similar results, and the findings of this study is in agreement with those (Bethell *et al.* 2012a; Watson, Ghodasra & Platt 2009; this study).

There is a strong potential explanation for the contradictory findings between human and macaque studies. Macaques live in strictly lineal hierarchies where males are dominant over females (Berman 1982). In the wild, males leave their natal group and join unrelated groups, where they will try to obtain mating opportunities either by building friendships with females or by attaining dominance through aggression (Qin *et al.* 2015). If an unknown conspecific male appears, it would be in the females best interest to 'lay low' in order to not be the target of his aggression as injuries are a great risk and impair her fitness (Lovell 1991). Direct eye contact is a threat in rhesus macaques and humans (Machado & Bachevalier 2008; Thatcher 2015; Helminen *et al.* 2011; Wieser *et al.* 2009) and gaze aversion is a signal of submission (Coss, Marks & Ramakrishnan 2002). Repeated eye contact with an aggressive unknown male would be unwise for a female. Further, the s-allele has been shown to enable individuals to avoid risks when they have the opportunity to (Homberg & Lesch 2011). Rhesus macaques can readily identify conspecifics and objects in photographs (Bovet & Vauclair 2000) but it is not known how readily they can decide that those are not real threats, hence, avoiding conflict caused by direct stare is a natural response. In humans, vigilance was linked to emotional vulnerability, whilst avoidance was linked to resilience and ll-homozygousity (Fox 1993; Beevers *et al.* 2007; Fox Ridgewell & Ashwin 2009). In macaques, s-allele carriers are able to avoid the aggressive conspecific face at a higher rate whilst ll-homozygous females are vigilant. I suggest this may be an advantage for s-allele carriers in normative circumstances, however, if s-allele carriers avoid factors they perceive as risks, they might end up avoiding a greater amount of factors than actually necessary. This increased avoidance would then present the same pattern of emotional vulnerability and resilience as suggested in humans.

Similar to the impact of the s-allele of *HTTLPR*, animals that did not carry a 7-repeat allele of the *MAOA* gene showed increased avoidance of the aggressive stimuli, whilst animals with one or two 7-repeats showed less avoidance. Sullivan *et al.* (2011) suggested that 77-homozygous females had higher scores in nervous temperament factors. It is possible that increased nervousness leads to females paying attention to the aggressive stimuli at a higher rate as a form of evaluating the situation, where insecurity and the danger of a potential attack increase nervousness. Interestingly, in humans, the low activity allele is thought to be connected to an impaired ability to control emotional responses during arousal (Meyer-Lindenberg *et al.* 2006) and in humans and macaques is linked to

impulsive behaviour (Gabel *et al*. 1995; Newman *et al.* 2005). Further, Newman *et al.* (2005) suggested an increased reactivity to stress in individuals carrying the 7-repeat allele. Such inability to control a response, impulsiveness and reactivity to stressful stimuli would show here in the form of low activity allele carriers being unable to control repeated vigilance towards an emotional stimulus, resulting in less avoidance of the aggressive stimulus.

The finding of nursing status and side was somewhat surprising. Firstly, nursing status alone had a significant impact, where females without a baby or a nursing baby were avoidant of aggressive stimuli, whilst females with an older infant were vigilant for those. Nursing status in combination with side showed a difference between females having no baby or nursing a baby compared to females with older infants. If females with older infants had the aggressive stimuli presented to their left they showed large variation and were not able to avoid the stimuli, whilst they avoided looking at the aggressive stimuli presented on their right at a higher rate as indicated by the error bars. However, females without a baby or with a nursing baby showed the opposite trend, avoiding aggressive stimuli presented on their left and being less avoidant when the aggressive stimuli were presented on their right. Side in general had a significant impact, where females overall tended to show more attention towards both stimuli if the aggressive stimuli were presented on their right, whilst they showed avoidance of the aggressive stimuli when they were presented on their left. This indicated that females were less able to avoid the aggressive stimuli presented to their right. In humans and macaques, a left gaze bias (LGB) has been found, which is linked to the right hemisphere of the brain, responsible for perceptual processing of facial information (Hamilton & Vermeire 1988; Hauser 1993; Butler *et al.* 2005). This means that the salience of information towards the left can be more readily evaluated, allowing for an increase of attention if necessary (Guo *et al.* 2009). The fact that macaques avoided aggressive stimuli towards their left at a higher rate indicates that they tended to evaluate the aggressive facial expression more readily and hence were able to avoid looking at it at a higher rate.

The reason why females with an older infant exhibited the opposite effect is unknown, but human error is a likely explanation. The sex of the offspring is unlikely to have an effect as, of the three females in this category, two had female offspring. However, a larger sample is needed in order to rule out this explanation completely. One possibility

however is, that it was influenced by location of testing. Although we aimed to station animals at different spots every time, this was not always possible due to hierarchy within the group and areas in which females were comfortable to station (*i.e*. access to escape routes for lower ranking females). Two of the three females were mostly tested at the left end (from the researchers point of view) of the caging area and hence, the part of the apparatus that was towards their right may have been obstructed by the end of the cage or not have been within the area that they would expect to encounter conspecifics, since the opposite cage area ended symmetrically. It might hence simply be a coincidence that two of the three females that fell into this nursing status category were tested at the end of the enclosure. Another possible explanation, which is not exclusive from the previous one, could be that they wanted to keep track of their infant in the social group and due to them being stationed at the end of the enclosure, their infant would be somewhere towards their left. They would not be able to avoid the aggressive stimuli when presented to their left as much as they might want to. Future testing should be carefully considered in terms of the position of the stationed individual in order to avoid this.

Impact of genotype on behaviour after presentation of social stimuli

These results should be interpreted with caution, as behavioural observations were carried out once all females in a group had been tested. This means, the female that was tested first had a longer break between testing and behavioural sampling than the female tested last. Immediate effects of the presentation of the stimuli might have been lost within those methods. However, it was not feasible to observe each female after testing and before testing the next one, as the social groups would have been unlikely to station for one trial only to then have to come back again within a short amount of time repeatedly.

In terms of impact of genotype on behaviours displayed after presentation of conspecific stimuli, there were several interesting findings. The *DRD4* T-allele carriers showed significantly higher rates of aggressive approach behaviour compared to AA-homozygotes. *MAOA* 7-repeat allele carriers showed increased affiliative approach and decreased maintenance behaviours compared to non-carriers. All those results were without application of Bonferroni and hence could be the result of multiple tests being carried out.

The finding on *DRD4* is particularly interesting as this was a newly identified SNP and this result could point to a possible impact of this genotype on behaviours related to aggression. *DRD4* has previously been linked to risk-taking and exploration (Coyne *et al.* 2015) as well as aggression towards conspecifics (Barr & Driscoll 2014) and aggressive behaviour yields a certain risk of getting into conflict. Findings of this *DRD4* SNP being involved in aggressive behaviour are supported by those previous findings and cement the role of dopamine in aggressive behaviour. Future studies should investigate whether the A>T SNP reported in this study has an impact on a variety of types of aggression or other behaviours. For example, *DRD4* in human and non-human primates has been shown to impact on novelty seeking (Ebstein *et al.* 1996; Bailey *et al.* 2007). In a comparison between two strains of rats, Walker *et al.* (2009) showed that a strain of rat displayed higher rates of novelty seeking as well as active coping styles compared to a different strain that displayed low novelty seeking and increased passive coping styles. Passive coping styles have been shown to be related to higher HPA axis reactivity (Koolhaas *et al.* 1999), higher plasma corticosterone levels (Korte *et al.* 1992) and psychopathology (Walker *et al.* 2008). Hence, novelty seeking is a trait that could easily be connected to welfare and coping styles of captive macaques and be impacted on by the *DRD4* polymorphism.

MAOA 7-repeat allele carriers showed increased affiliative approach and decreased maintenance behaviours compared to non-carriers. The finding that the low activity allele was linked to increased affiliative approach behaviours such as grooming and lip smacks contradicts those of increased aggression, impulsivity and antisocial behaviour in humans and macaques mentioned here previously (Barr *et al.* 2004c). Females carrying the low activity allele, who are known to react more to stress (Newman *et al.* 2005), were possibly trying to counteract their stress response by engaging in affiliative, reassuring contact with their social group after having seen the aggressive males face. Grooming in primates strengthens bonds and friendships between animals (Massen & Sterck 2013; Puga-Gonzalez, Hoscheid & Hemelrijk 2015) and the increase in affiliative behaviour might present an active coping style of females to handle stress (Cheney & Seyfarth 2009). In fact, although some individuals are under increased risk of stress within challenging situations, they also respond better to supportive environments (Kaufmann *et al.* 2004; Beaver & Belsky 2012). If this is the case, then the presence of conspecifics is imperative to allow for such coping to take place and macaques that are exposed to stress on a

regular basis should not be housed alone (Baker *et al.* 2012; Wolfensohn & Honess 2005). The decrease in maintenance behaviour could be linked to an increase in stress reactivity and inability to control their emotional response (Newman *et al.* 2005; Meyer-Lindenberg *et al.* 2006). Females might not be able to perform maintenance behaviours, such as foraging or resting after seeing emotion-eliciting stimuli that caused a stress response because they are too wound up and unable to control their emotional response.

Some non-significant findings indicated that the *HTTLPR* s-allele led to increased aggressive approach behaviours and decreased fear-avoid behaviours. Both those results go well together, as an animal that shows increased aggression towards conspecifics is unlikely to show fear and submissive behaviours at the same time. The s-allele has been linked to aggression in previous studies (Barr & Driscoll 2014) and the finding here supports those. If presentation of emotionally valenced stimuli elicits an aggressive response, the way attention bias testing is carried out has to be considered carefully in order to not cause unnecessary stress to the social group following testing.

Chapter 4. Testing the effectiveness of veterinary habituation through attention bias testing and the impact of genotype on attention bias for an emotional stimulus: veterinarian vs pixelated face (Experiment 2)

This chapter starts with a brief introduction to the experiment and describes the methods for the attention bias testing, using photographs of the facilities' veterinarian. I explain the different types of habituation to the vet that animals underwent and the statistical analysis carried out. I then present the results in regards of genotype impact on attention bias. Further, the results section includes the results from behavioural observations and genotype impact on behaviour following the attention bias tests and the impact of genotype on success of habituation. Overall success of habituation, regardless of genotype but in regard of the amount of habituation received, is also assessed. At the end of the results, behaviours were compared between the two different types of stimuli presented in this study in order to assess stimuli-specific behavioural changes. All those results are discussed.

Introduction

Welfare of captive animals and rhesus macaques is of great importance (NC3Rs 2011). However, there are factors in captivity that cause stress to animals, which cannot be avoided. Such factors can be regular invasive procedures such as drawing blood as well as veterinary checks and interventions (Bethell *et al.* 2012b) or even just slight changes in the physical or social environment (Suomi 2006). Veterinarians are what I here describe as an aversive stimulus. Upon arrival of a veterinarian, not only the animal he/she is investigating, but often the entire group of animals react in an excited, agitated manner (*pers. obs*.). As animals associate the veterinarian with possible painful, invasive procedures, it is unlikely, and in this study assumed, that they are seen as a positive stimulus.

As mentioned in Chapter 1, genotype has been found to have impacts on reward-related behaviours and hence I hypothesized that it would also impact on responsiveness to habituation, where some indivudals would habituate to the veterinarian at a higher level compared to others. This was measured by comparing their vigilance/ avoidance levels of

the veterinarian stimulus before and after the habituation took place. The veterinarian at CFM agreed to take part in a habituation program with several social groups which gave way to apply the AB paradigm in order to measure how the cognitive response to the veterinarian changes with habituation. A program which can be implemented in a range of captive environments has to be designed on a realistic concept that is managable for all individuals involved. Veterinarians and care staff are extremely busy and usually have little extra time; hence this study was focused on only a fraction of all individuals housed at CFM and using habituation sessions of only five minutes every other week. Further, I aimed to investigate whether females treat each veterinarian as an individual, or generalise them all as one. By having females which received one session of habituation with the veterinarian of whom they saw the stimuli and one other facility veterinarian I wanted to see whether their AB would change.

Further, current methods of assessment for habituation rely on observational measures of behaviour but not cognitive ones. Using AB to assess this hence presented a new way of confirming what has been shown with behavioural observations as well as investigating whether the AB paradigm is capable of detecting changes in the cognitive perception of a stimulus.

Materials and Methods

4.1 Animals, Housing and Apparatus

Twenty-five females of the twenty-nine for whom genetic data were available took part in Experiment 2, 24 of those were the same as in Experiment 1. Mean (±SE) age was 9.75 years (±3.41) and a full list of their matrilines can be found in Appendix 8. Housing conditions, stations of individuals and social grouping were the same as in Experiment 1, as was the apparatus.

4.2 Stimuli

Stimulus pairs included an image of one of the facility's veterinarian (threat) vs. a pixelated, luminance- and size matched version of the same image (non-threat, Fig. 4.1), resembling the colour pattern of the photograph of the vet. All stimuli were approximate life-size printed on A4 paper. The pixelated version was created using Appendix 9 shows all stimuli pairs used in this study. Those were followed by presentation of unknown, conspecific infants in order to avoid that monkeys perceived attention bias testing as negative *per se* (Fig 4.2).

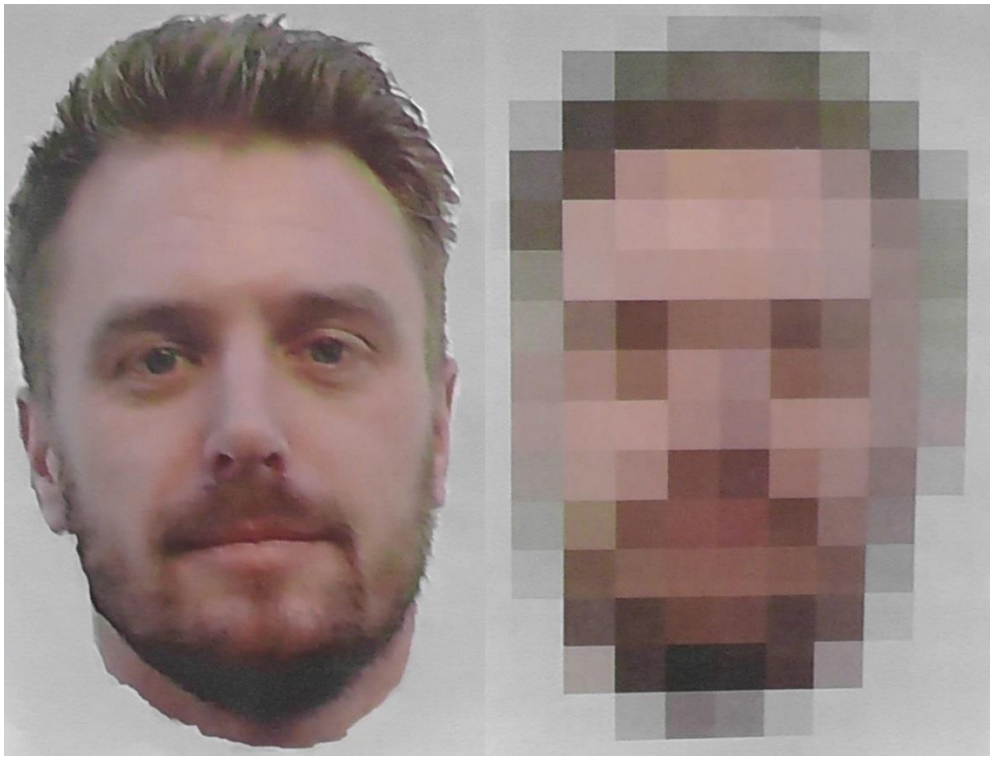

Figure 4.1
Example stimuli pair of the facility veterinarian and the same photograph as a pixelated, luminance matched version. All stimuli faced inwards. Photograph of the facility veterinarian used and published with his consent.

Figure 4.2
Example stimuli pair of unknown, conspecific infants used as positive stimuli for the attention bias testing of rhesus macaques, *Macaca mulatta*. Photographs of stimuli taken by CFM staff and used with their consent.

4.3 Procedure

Attention bias testing followed the same procedure as in Experiment 1. Females received four presentations of stimulus pairs lasting three seconds each as counted by the researcher. The first two presentations took place within one week, no longer than three days apart, followed by a break of roughly 12 weeks after which they were presented with the third and fourth presentation within five days. During the 12 weeks of no testing, habituation to the veterinarian occurred (see below). Once all females had been tested, behavioural observations were carried out as described in Experiment 1.

4.4 Habituation conditions

Females in Experiment 2 were split into three groups: the control group (n=8) received no habituation to the veterinarian; the regular habituation group (n=10) received five minutes of habituation in the form of hand feeding of raisins and peanuts by the veterinarian fortnightly resulting in an overall number of six habituation sessions; and the irregular habituation group (n=7) which received two opportunistic sessions of habituation, one with the veterinarian whose face was used in the attention bias testing and the other session with one of the other facility veterinarians.

4.5 Video coding

Videos of the attention bias trials were blind-coded for gaze towards the stimuli as in Experiment 1. Videos were also coded for behavioural responses to the veterinarian stimuli during the testing. The following four behaviours were noted during the presentation of stimuli as either occurring (1) or not occurring (0): fear grin, lip smack, alarm bark and flee. Those behaviours were chosen as they were performed at a high rate by the females during attention bias testing of those stimuli. A "fear score" was calculated by adding the sum of all those four categories for each trial. On a scale of 0-4, this resulted in a maximum "fear score" of 4 for each presentation.

4.6 Statistical analysis of effect of genotype on AB

Associations between attention bias and genotypes either at individual loci or at the multi-locus level were examined in the *R Statistical software* package (version 3.2.0, R Core Team 2015), using generalised linear mixed effects models (GLMM) and the lme4 (Bates *et al.* 2015) and MuMIn (Barton 2015) packages. Genotypes were classed into two categories as explained in Experiment 1.

To investigate whether genotype had an impact on attention bias, all recorded explanatory variables needed to be controlled for. Those variables were the same as in Experiment 1. The cbind command was used as in Experiment 1 and models were selected on AICc values as explained previously.

Preliminary analysis for collinearity showed that age correlated with previous exposure (r=0.43, p<0.001). Therefore previous exposure was not included in the analysis.

As in Experiment 1, to control for relatedness between individuals, monkey identity (MonkeyID) was nested in matriline (Matriline). The amount of presentations (Presentation) a female had at the point of testing was included as a random factor and matriline and monkey ID were included as nested random factors. Age (AgeMos), group (Group), nursing status (NursStat) and the side (Side) on which the negative stimulus was presented were also included as fixed factors, resulting in the following maximum model:

glmer(AB ~ AgeMos + Group + NursStat + Side + (1|Presentation) + (1|Matriline/MonkeyID), family=binomial)

Thirty candidate models (Appendix 10) with binomial error structure were created from this maximum model and based on the lowest AICc value, the best fit model out of those that were not over-parametrised was selected.

To test for an effect of fixed factors from the best-fit models in combination with genotypes, test models were created which included each fixed factor from the previous best-fit models in combination with the genotypes (with random factors held constant). The following model was the maximum model:

glmer(AB ~ Side + AgeMos + HTTLPR + TPH2 + MAOA + OPRM1 + DRD4 + (1|Presentation) + (1|Matriline/MonkeyID), family=binomial)

To investigate whether *HTTLPR* and *TPH2* in combination had a larger effect (Brent *et al.* 2013), the best fit models from the above list were compared to ones that included the new variable *HTT-TPH*. AIC scores were the exact same for the combination of *HTTLPR* and *TPH2* as for *TPH2* alone.

When looking at a best fit model, there is a potential for variables not being significant (Crawley 2007). Weighted regression analysis was then used to select the best model by carrying out a deletion test. To do this, a simpler model was created, missing the least significant variable of the previous model. A Chi-squared test by running an ANOVA between the two models was carried out (Crawley 2007). As the response variables were binomial data, a binomial error structure was used. A *p*-level of $p \leq 0.05$ was used for significance.

4.7 Statistical tests for impact of genotype on behaviours and habituation

Differences in behaviour during the attention bias experiment as well as the behavioural observations following it, were examined in SPSS (version 22). Behavioural data were classed into five distinctive categories, as seen in Experiment 1. A Shapiro Wilk test was run to assess normality of the different behavioural categories (Appendix 11) and appropriate tests were used.

To test whether there was an overall difference between the pre- and post-habituation values of all animals, a Wilcoxon-signed rank test was run for aggressive-, affiliative- and fear behaviour and a Paired-samples *t* test for self-directed- and maintenance behaviour.

A Mann-Whitney *U* test (Independent-samples *t* test for self-directed and maintenance) was run to investigate whether the two genotype classes had an effect on the type of behaviours displayed and whether the different habituation groups displayed differences in behaviour post-habituation. A Wilcoxon-Signed rank test (Paired-samples *t* test for self-directed and maintenance) was run to compare each group pre- vs. post-habituation in the behavioural categories displayed. In order to investigate whether genotype class had an impact on the success of habituation and whether success of habituation was varying between specific habituation groups Paired-samples *t* tests were run to compare the attention bias scores of individuals. Whether there was a significant difference in the amount of fear behaviours recorded during attention bias testing was tested by running a Wilcoxon-Signed rank test. Lastly, a test was performed to compare whether females displayed differences in behaviours between Experiment 1 and Experiment 2. Data of the two experiments were treated as dependent data, as 24 of the 29 females were the same in both studies and a Wilcoxon-Signed rank test (Paired-samples *t* test for self-directed and maintenance) was performed.

4.6 Effect of genotype on attention bias: veterinarian *vs* pixelated face

When *MAOA* was classed conservatively (see methods) with all females carrying one or more 7-repeat alleles in one group, the best fit model included age, side, *HTTLPR, TPH2*, *OPRM1* and *DRD4* as separate factors. A new list of models investigated whether any of those factors in interaction explained the attention bias data better than just each factor separate. The best fit models from this model selection showed an interaction between side and age, *HTTLPR* and *TPH2* in combination, *OPRM1* and *DRD4.* The second best model included an interaction between side and age, *HTTLPR*, *TPH2*, *OPRM1* and *DRD4* (Table 4.5). The best fit model still met the assumptions of normality (Appendix 12).

Table 4.5. AIC_c - ranked candidate models showing the relative importance of fixed effects (side, age, nursing status, *HTTLPR* and *TPH2* combined, *OPRM1, DRD4, MAOA, TPH2*) and random effects (presentation, monkey ID and matriline) in explaining attention bias of female rhesus macaques, *Macaca mulatta*.

Fixed effects	Random effects (/nested)	d.f.	Log likelihood	AIC	delta
Side x Age, *HTTLPR* and *TPH2* combined, *DRD4*, *OPRM1*	(Matriline/ MonkeyID), Presentation	44	-7854.269	15868.5	0.00
Side x Age, *HTTLPR*, *TPH2*, *DRD4*, *OPRM1*	(Matriline/ MonkeyID), Presentation	45	-7966.086	16098.8	230.3

4.7 Weighted regression analysis

To ascertain that this model did in fact explain AB data better than any other model, further model selection was carried out. The current best fit model was compared to a second model, which did not include the previous least significant factor and those two models were then compared by running an *ANOVA*. If the *ANOVA* was not significant, the original model was not significantly better at explaining AB data compared to the newly designed one. If this was the case, the next least significant factor was removed and again, models were compared with an *ANOVA*, until the *ANOVA* yielded a significant result, indicating that the previous model explained the AB data significantly better than the newly designed one.

All factors besides the interaction of age and side as well as *HTTLPR* and *TPH2* in interaction were removed during the weighted regression analysis. The interaction of age and side had a significant impact on attention bias (Table 4.6, Fig. 4.4). Females aged between 2.5 and 7.5 years showed the least avoidance of threat stimuli presented to their left hand side whilst females between 11.6 and 15.7 years showed the greatest avoidance of threat stimuli overall, especially when presented on their right hand side compared to the other two age classes. Age was not significant by itself whilst side alone was strongly significant (Table 4.6, Fig. 4.5). Further, the interaction between *HTTLPR* and *TPH2* was significant where females carrying combination 1 genotypes (see Chapter 3) showed greater avoidance of the vet threat stimuli compared to combination 2 carriers (Fig. 4.6).

Table 4.6. The best fit model and the *z*- and *p*- values for impact on attention bias of female rhesus macaques, *Macaca mulatta*. Significance assigned at $p \leq 0.05$*, $p \leq 0.005$**, $p \leq 0.001$***.

Factor	*z*-value	*p*-value
Age x Side	*z*=-17.82	*p*<0.001***
Age	*z*=-1.38	*p*=0.168
Side	*z*=38.24	*p*<0.001***
***HTTLPR* and *TPH2* combined**	*z*=-9.87	*p*<0.001***

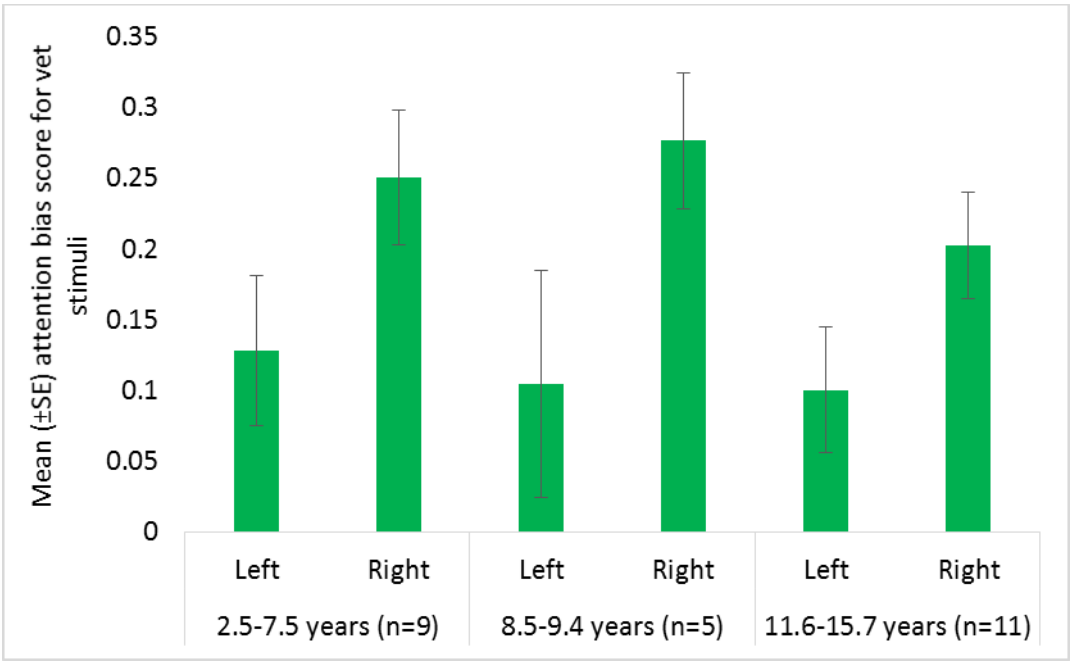

Figure 4.4
Mean (±SE) attention bias score of rhesus macaque, *Macaca mulatta*, females (n=25) for the threat and non-threat vet stimuli, depending on their age and the side on which the threat stimuli were presented to them. Ages 2.5-7.5 included 9 females, 8.5-9.4 years included 5 females and

ages 11.6-15.7 included 11 females. Positive values indicate vigilance for- and negative values avoidance of the threat stimuli.

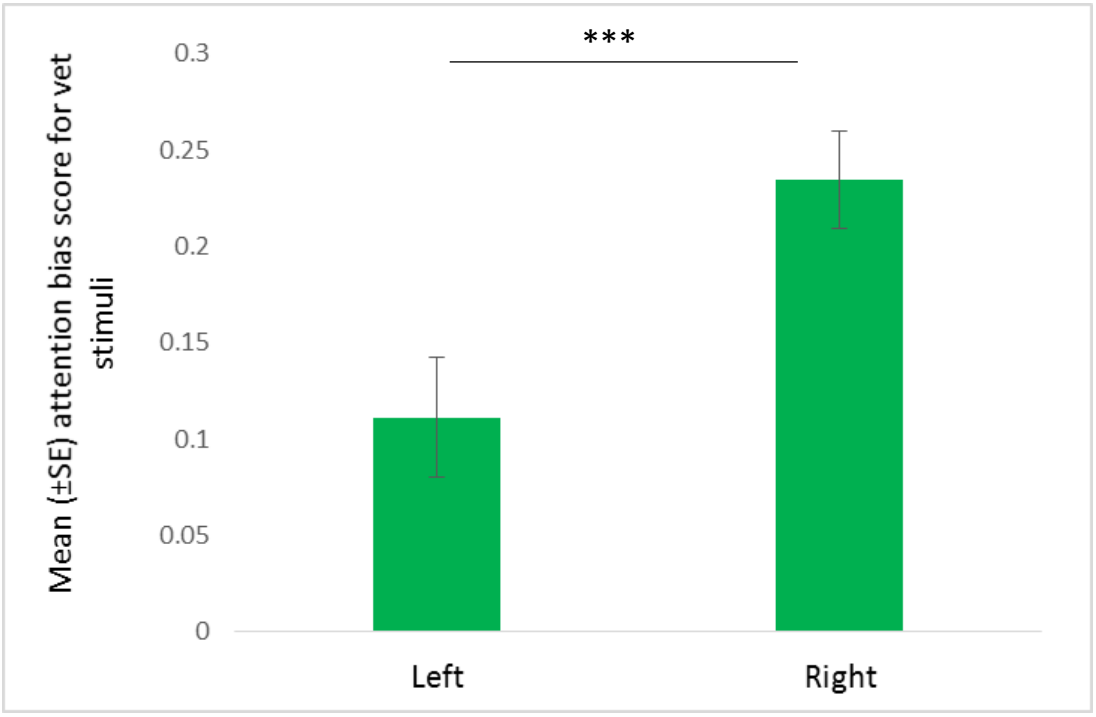

Figure 4.5
Mean (±SE) attention bias score of rhesus macaque, *Macaca mulatta*, females (n=25) for the threat and non-threat vet stimuli, depending on the side on which the threat stimuli were presented to them. Positive values indicate vigilance for- and negative values avoidance of the threat stimuli. Significance assigned at $p \leq 0.05$*, $p \leq 0.005$** and $p \leq 0.001$***.

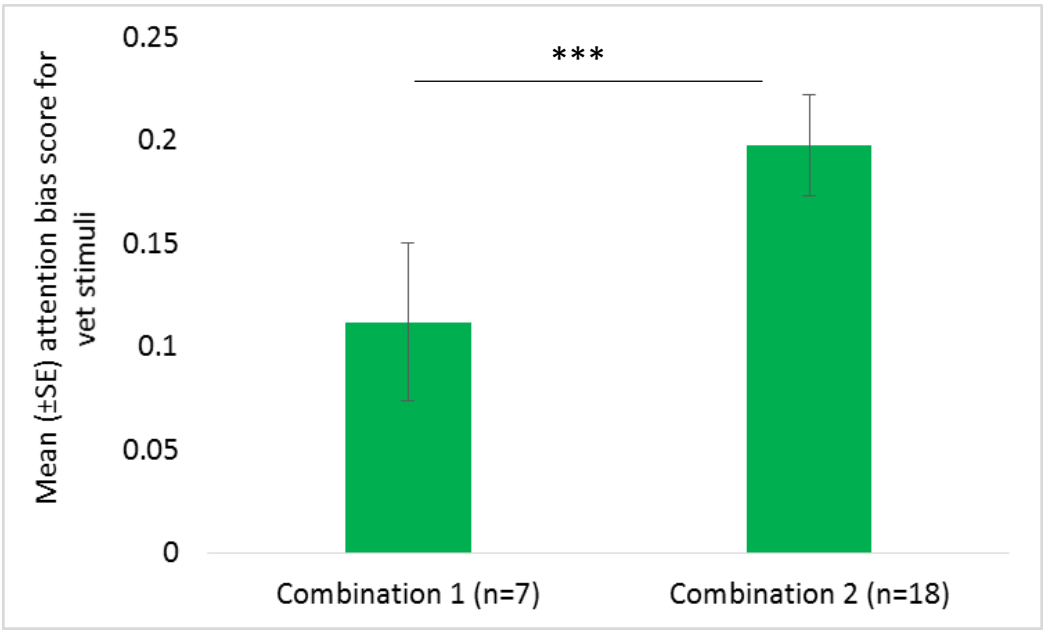

Figure 4.6
Mean (±SE) attention bias score of rhesus macaque, *Macaca mulatta*, females (n=25) for the threat and non-threat vet stimuli, depending on the combination of *HTTLPR* and *TPH2* genotype (Combination1: *HTTLPR* ll-homozygous and *TPH2* ss-homozygous (n=7); Combination 2: One genotype homozygous the other heterozygous (n=18); Combination 3: *HTTLPR* ss-homozygous and *TPH2* ll-homozygous (n=0)). Positive values indicate vigilance for- and negative values avoidance of the threat stimuli. Significance assigned at $p \leq 0.05$*, $p \leq 0.005$** and $p \leq 0.001$***.

MAOA could not be assessed by grouping 77-homozygous individuals against all others, as only one female was 77-homozygous.

4.8 Was there an overall difference in the behaviours females displayed after presentation of the vet stimuli during the pre- and post-habituation trials?

There were no significant differences between the behavioural categories pre- and post-habituation (Table 4.7).

Table 4.7. *z*- or *t*-values (d.f.) and the *p*-values of Wilcoxon Signed rank and Paired samples *t*-tests for differences between behavioural categories pre- and post-habituation after attention bias testing of female rhesus macaques, *Macaca mulatta*.

Genotype	*z*-value/ *t*-value (d.f.)	*p*-value
Aggressive	-1.647	0.1
Affiliative	-1.478	0.139
Fear	-0.408	0.683
Self-directed	-0.792 (24)	0.436
Maintenance	0.022 (24)	0.983

4.9 Did genotype generally affect the type of behaviours displayed by females?

There was no significant difference between the two genotype classes in any of the behavioural categories displayed (Table 4.8).

Table 4.8. *U*- or *t*-values (d.f.) and *p*- values of Mann-Whitney *U* tests and Independent samples *t*-tests on impact of genotype classes on behaviours performed by female rhesus macaques, *Macaca mulatta,* after attention bias testing. These results are presented without the corrected *p*-value for Bonferroni.

Behaviour	Genotype	*U*-value	*p*-value
Aggressive	*HTTLPR*	47.5	0.098
	TPH2	60.5	0.669
	MAOA conservative	50	0.315
	OPRM1	15	0.480
	DRD4	49	0.423
	HTTLPR and *TPH2* combined	32.5	0.243

Affiliative	HTTLPR	42	0.052
	TPH2	50	0.315
	MAOA conservative	59	0.628
	OPRM1	13	0.373
	DRD4	52	0.534
	HTTLPR and TPH2 combined	47	0.869
Fear	HTTLPR	73	0.810
	TPH2	60	0.669
	MAOA conservative	61	0.711
	OPRM1	19	0.733
	DRD4	58	0.790
	HTTLPR and TPH2 combined	43	0.668
Self-directed	HTTLPR	1.596 (23)	0.124
	TPH2	0.614 (23)	0.545
	MAOA conservative	-0.007 (23)	0.995
	OPRM1	-0.124 (23)	0.903
	DRD4	1.219 (23)	0.235
	HTTLPR and TPH2 combined	0.158 (23)	0.876
Maintenance	HTTLPR	-1.097 (23)	0.284
	TPH2	0.510 (23)	0.615
	MAOA conservative	-0.184 (23)	0.855
	OPRM1	-0.572 (23)	0.573
	DRD4	-0.703 (23)	0.489
	HTTLPR and TPH2 combined	0.579 (23)	0.568

The two non-significant trends for the *HTTLPR* genotype classes (Table 4.8) showed that females carrying ≥1 s-allele displayed less aggressive behaviours (Fig. 4.7) and more affiliative behaviours (Fig.4.8) compared to ll-homozygous females.

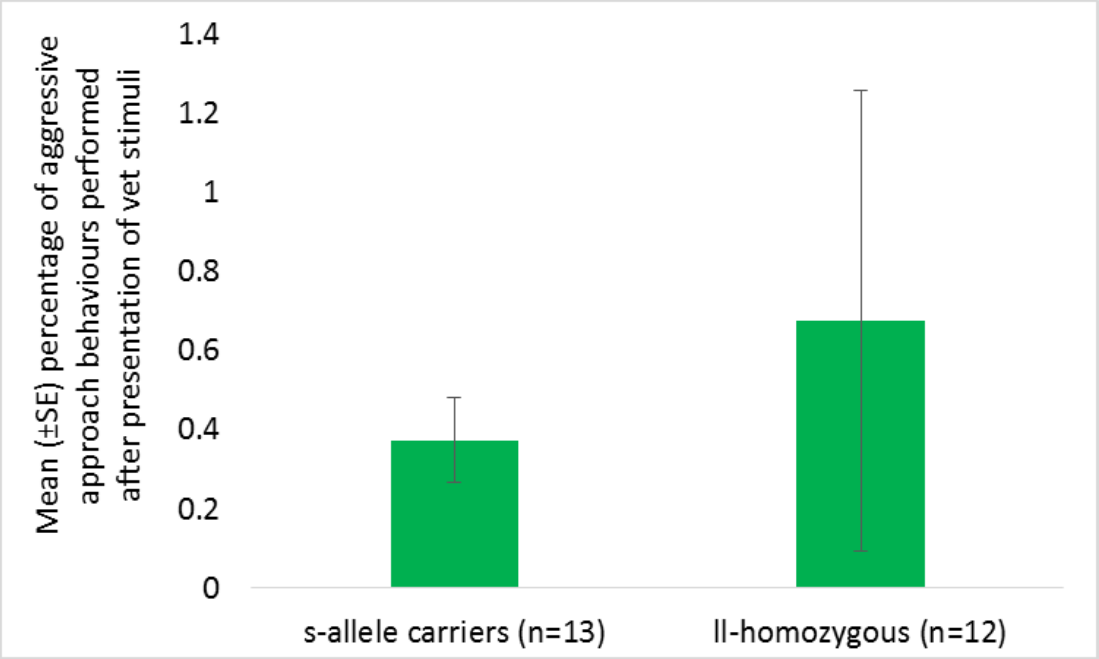

Figure 4.7
Mean (±SE) percentage of aggressive approach behaviours displayed by rhesus macaque, *Macaca mulatta*, females (n=25) after presentation of threat (vet) and non-threat (pixelated) stimuli, depending on their *HTTLPR* genotype (ll-homozygous (n=12), s-allele carriers (n=13)).

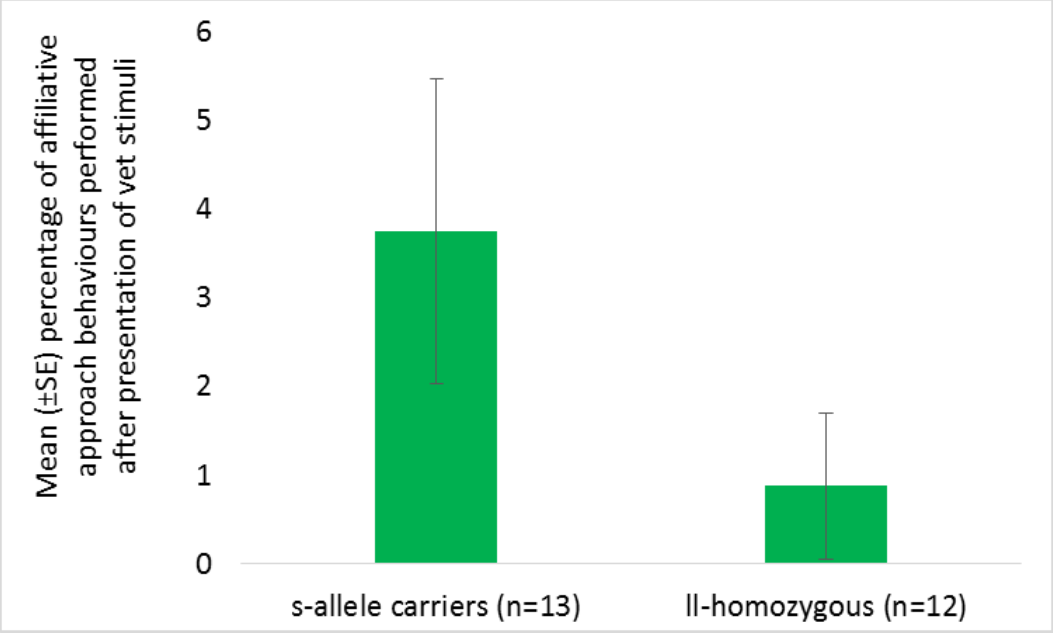

Figure 4.8
Mean (±SE) percentage of affiliative approach behaviours displayed by rhesus macaque, *Macaca mulatta*, females (n=25) after presentation of threat (vet) and non-threat (pixelated) stimuli, depending on their *HTTLPR* genotype (ll-homozygous (n=12), s-allele carriers (n=13)).

4.10 Did genotype have an effect on the success of habituation?

There were no significant differences between genotype classes on attention bias score post habituation (Table 4.9).

Table 4.9. *t*-values (d.f.) and *p*-values for impact of genotype classes on attention bias scores of threat and non-threat vet stimuli post-habituation by 25 female rhesus macaques, *Macaca mulatta*, as measured by an Independent-samples *t* test.

Habituation group	Gene	*t*-value (d.f.)	*p*-value
Control	*HTTLPR*	-1.113 (14)	0.485
	TPH2	1.74 (14)	0.104
	MAOA conservative	1.425 (14)	0.176
	OPRM1	1.127 (14)	0.279
	DRD4	1.035 (14)	0.318
	HTTLPR and *TPH2* combined	No variation	
Irregular habituation	*HTTLPR*	0.825 (12)	0.426
	TPH2	No variation	
	MAOA conservative	-1.783 (12)	0.1
	OPRM1	No variation	
	DRD4	-0.05 (12)	0.961
	HTTLPR and *TPH2* combined	-1.145 (12)	0.275
Regular habituation	*HTTLPR*	-0.264 (18)	0.798
	TPH2	0.508 (18)	0.617
	MAOA conservative	0.448 (18)	0.659
	OPRM1	-0.443 (18)	0.663
	DRD4	-1.837 (18)	0.083
	HTTLPR and *TPH2* combined	0.798 (18)	0.435

DRD4 showed a non-significant trend in the regular habituation group, where T-allele carriers were more avoidant of the threat vet stimuli post habituation compared to AA-homozygous females (Table 4.9, Fig. 4.9).

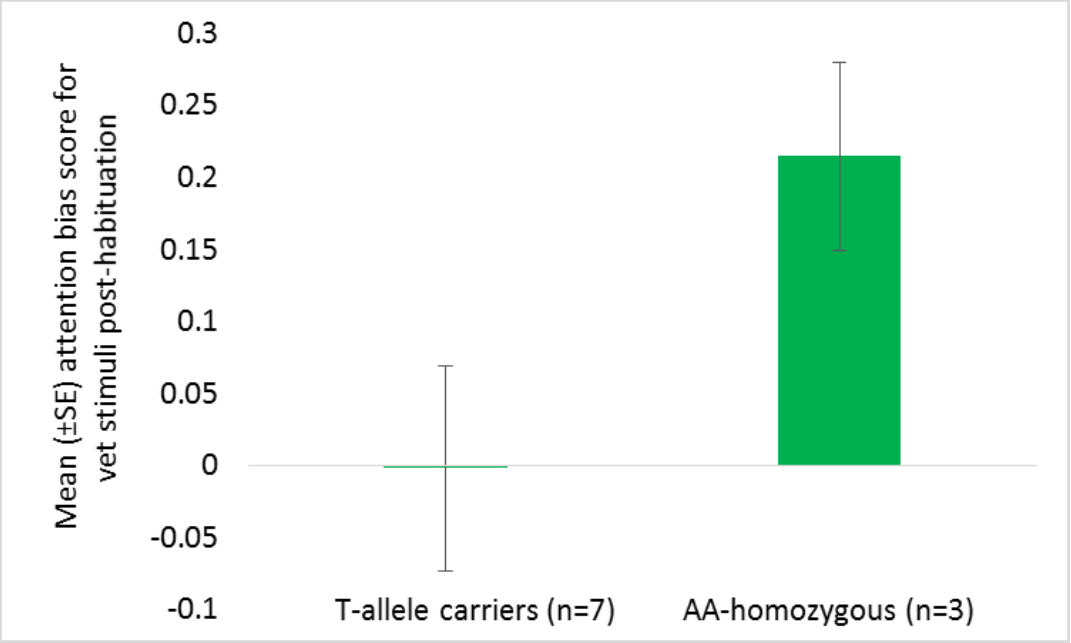

Figure 4.9
Mean (±SE) attention bias score for threat and non-threat stimuli by rhesus macaque, *Macaca mulatta*, females after having received regular habituation (n=10) depending on their *DRD4* genotype (T-allele carriers (n=7), AA-homozygous (n=3)). Positive values indicate vigilance for- and negative values avoidance of the threat stimuli.

4.11 Was there an overall difference between pre- and post-habituation of attention bias for the vet stimuli?

There was a strong significant difference between the attention bias score pre- compared to post habituation in all females, with animals being less vigilant for the vet threat-stimuli post habituation (Paired-samples *t* test, t_{49}=3.44, *p*=0.001, Fig 4.10).

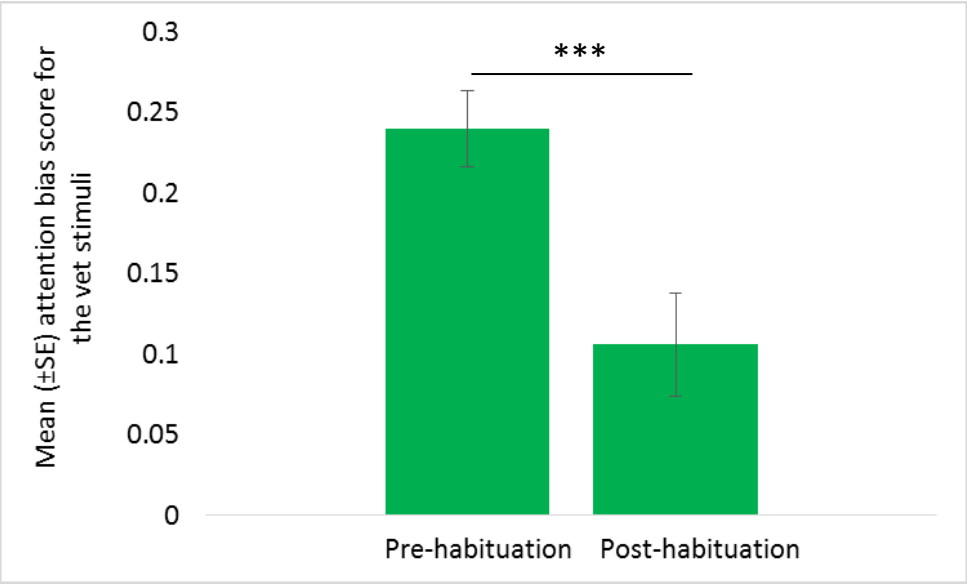

Figure 4.10

Mean (±SE) attention bias score of all groups of female rhesus macaques, *Macaca mulatta*, (n=25) for the threat and non-threat stimuli pre- and post-habituation. Significance was assigned at the p=0.05 level where * p≤0.05, **p≤0.005, ***p≤0.001.

4.12 Was there a difference of attention bias score for vet stimuli before and after habituation within each habituation group?

There was no significant difference in attention bias score within the control group pre- and post-habituation (Table 4.10). Their attention bias score was less vigilant for the threat stimuli after a period of 12 weeks but this was non-significant (Fig. 4.11). There was a significant difference within the irregular habituation group (Fig 4.1) and within the regular habituation group between the attention bias scores pre- and post-habituation (Table 4.10, Fig. 4.1). Females looked at the threat stimuli more pre-habituation compared to post-habituation.

Table 4.10. *t*- values (d.f.) and *p*-values for Paired samples *t*-tests of impact of habituation on attention bias in female rhesus macaques, *Macaca mulatta*. Significance assigned at *p*≤0.05*.

Habituation group	*t*-value (d.f.)	*p*-value
Control (n=8)	0.854 (15)	0.407
Irregular habituation (n=7)	2.401 (13)	0.032*****
Regular habituation (n=10)	2.692 (19)	0.014*****

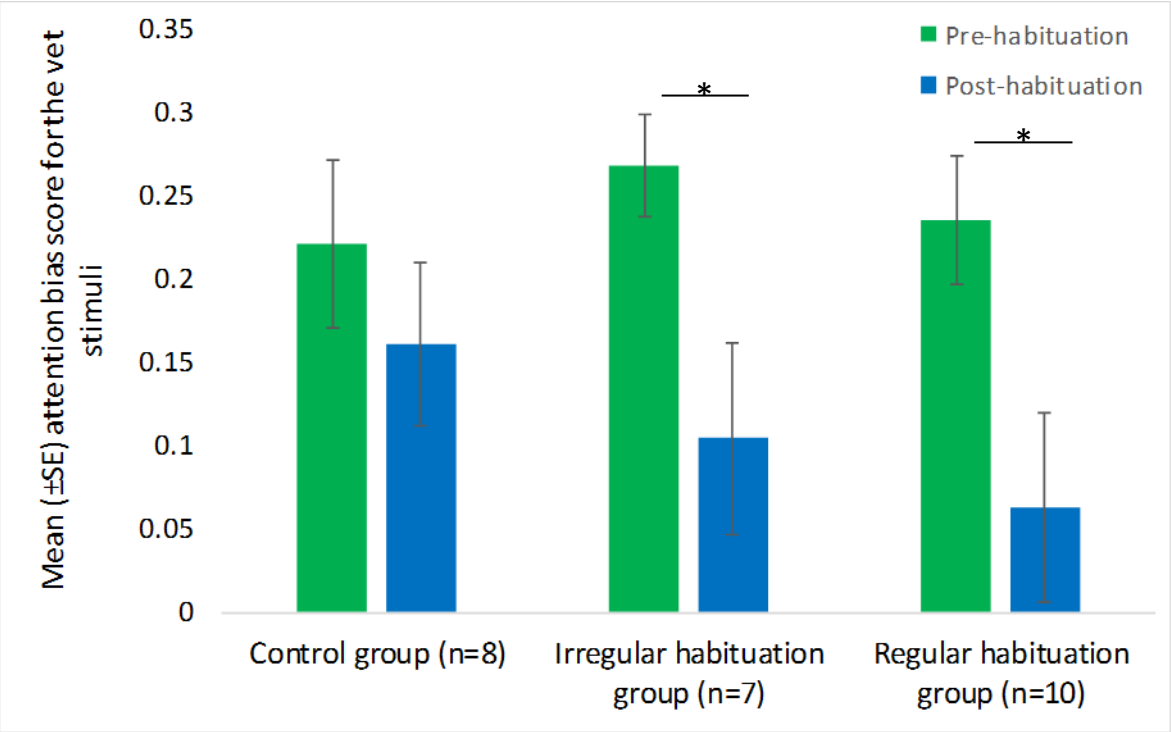

Figure 4.11
Mean (±SE) attention bias score of female rhesus macaques, *Macaca mulatta*, of the control group (n=8), the irregular habituation group (n=7) and the regular habituation group (n=10) for the threat and non-threat vet stimuli pre- and post-habituation.

4.13 Was there a difference in behaviours displayed between the different habituation groups post-habituation?

There was no significant difference between the amount of behaviours displayed post-habituation between the control group and the irregular-habituation group or the irregular-habituation group and the regular-habituation group (Table 4.11). For the non-significant effects the irregular habituation group displayed more aggressive behaviours on average compared to the control group (Fig. 4.12) whilst the control group displayed more maintenance behaviours on average compared to the irregular-habituation group (Fig. 4.13).

There was a significant difference between the control group and the regular habituation group, with the regular habituation group displaying significantly more aggressive behaviours post-habituation (Table 4.11, Fig. 4.14). This did not withstand Bonferroni correction. There were no other significant differences between the control group and the regular-habituation group (Table 4.11). All those results reported are without Bonferroni correction.

Table 4.11. *U*- or *t*-values (d.f.) and *p*-values for Mann-Whitney *U* tests and Independent samples *t*-tests of impact of habituation on attention bias in female rhesus macaques, *Macaca mulatta,* before Bonferroni correction. Significance assigned at $p \leq 0.05$*.

Habituation groups	Behaviour	*U*-value/ *t*-value (d.f.)	*p*-value
Control *vs.* Irregular-habituation	Aggressive	12	0.072
	Affiliative	20	0.397
	Fear	24.5	0.694
	Self-directed	-1.203 (13)	0.251
	Maintenance	2.029 (13)	0.063
Irregular-habituation *vs.* Regular-habituation	Aggressive	33.5	0.887
	Affiliative	32	0.813
	Fear	34	0.962
	Self-directed	1.458 (15)	0.165
	Maintenance	-1.509 (15)	0.152
Control *vs.* Regular-habituation	Aggressive	12	0.012*
	Affiliative	27.5	0.274
	Fear	38	0.897
	Self-directed	0.158 (16)	0.876
	Maintenance	0.136 (16)	0.893

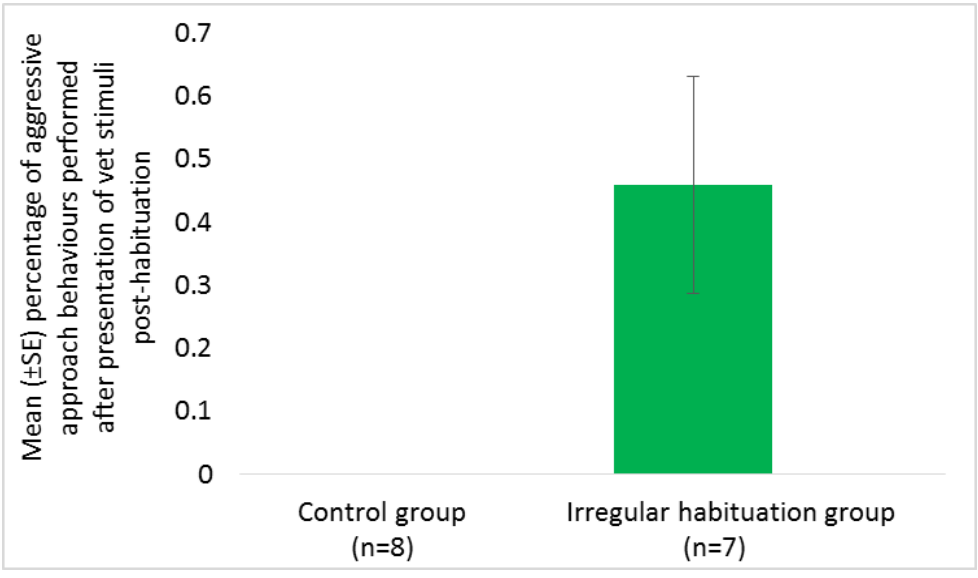

Figure 4.12
Mean (±SE) percentage of aggressive behaviours displayed by rhesus macaque, *Macaca mulatta*, females after presentation of vet stimuli post-habituation for the control group (n=8) and the

irregular habituation group (n=7). The control group did not perform aggressive approach behaviours.

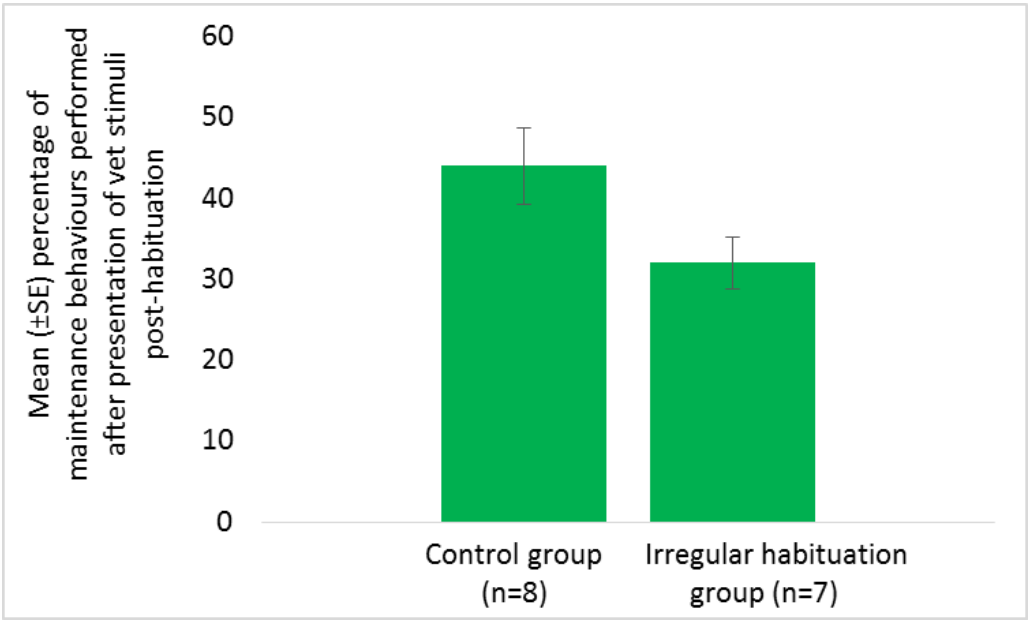

Figure 4.13
Mean (±SE) percentage of maintenance behaviours displayed by rhesus macaque, *Macaca mulatta*, females after presentation of vet stimuli post-habituation for the control group (n=8) and the irregular habituation group (n=7) post habituation.

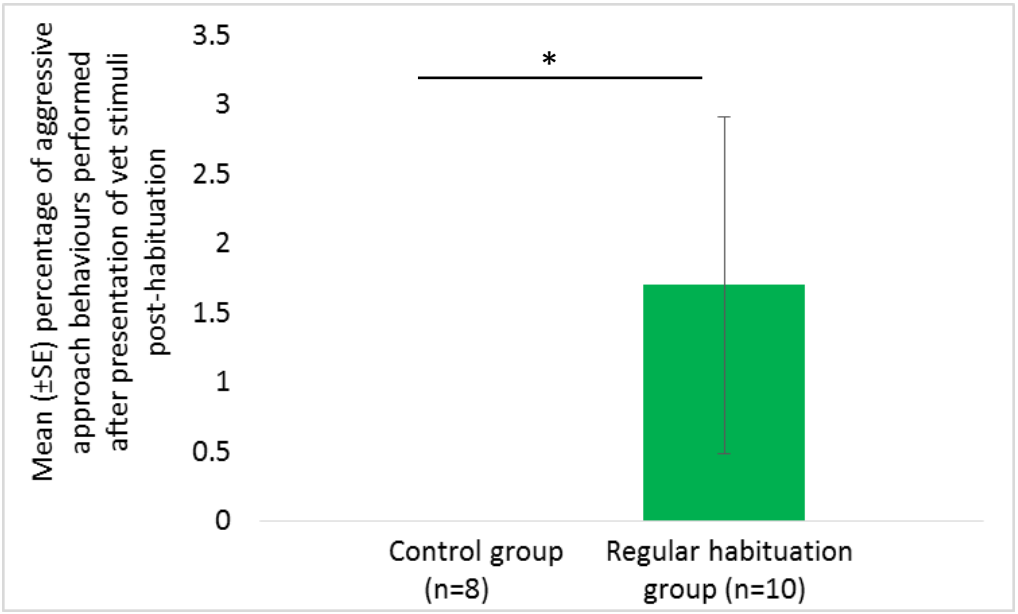

Figure 4.14
Mean (±SE) percentage of aggressive behaviours displayed by rhesus macaque, *Macaca mulatta*, females after presentation of vet stimuli post-habituation for the control group (n=8) and the regular habituation group (n=10).Significance assigned at p≤0.05*.

4.14 Was there a significant difference in fear behaviours recorded during presentation of the stimuli pre- and post-habituation?

There was no significant difference in the amount of fear behaviours displayed pre- and post-habituation in the control group or in the irregular habituation group (Table 4.12). There was a significant difference in the amount of fear behaviours displayed whilst being presented with the vet stimuli pre- and post-habituation in the regular habituation group (Table 4.12, Fig. 4.15). Females showed higher amounts of fear behaviours before than after habituation.

Table 4.12. *z*- and *p*-values of a Wilcoxon Signed rank test between fear behaviours performed during attention bias testing of female rhesus macaques, *Macaca mulatta*, pre- and post-habituation, depending on the amount of habituation they received. Significance assigned at $p \leq 0.05$*.

Habituation group	*z*-value	*p*-value
Control (n=8)	-1.552	0.121
Irregular habituation (n=7)	-1.535	0.125
Regular habituation (n=10)	-2.333	0.020*****

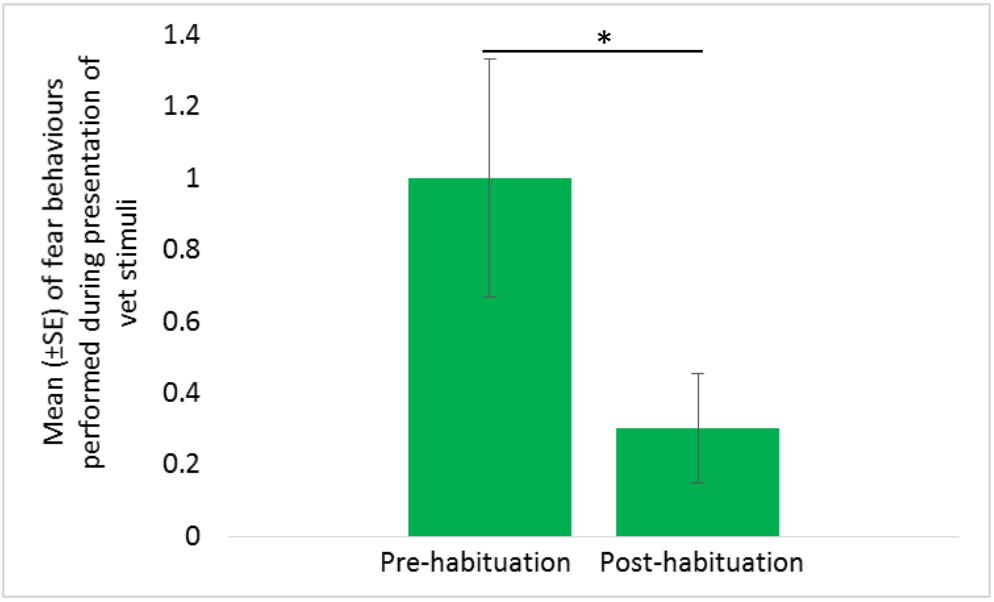

Figure 4.15
Mean (±SE) percentage of fear behaviours the regular habituation group of female rhesus macaques, *Macaca mulatta*, (n=10) displayed during presentation of the vet stimuli pre- and post-habituation. Significance was assigned at the p=0.05 level where p≤0.05*.

4.15 Was there a significant difference between the amounts of fear behaviours recorded during presentation of the stimuli post-habituation between the three habituation groups?

There was no significant difference in the amount of fear behaviours displayed between the control and the irregular habituation group, between the irregular and the regular habituation group, or the control and the regular habituation group (Table 4.13).

Table 4.13. *U*- and *p*-values of a Mann-Whitney *U* test between fear behaviours performed during attention bias testing of female rhesus macaques, *Macaca mulatta*, post-habituation, depending on the amount of habituation they received.

Habituation groups	*U*-value	*p*-value
Control *vs*. Irregular habituaiton	24.5	0.694
Irregular habituation *vs.* Regular habituation	27.5	0.475
Control *vs.* Regular habituation	22.5	0.122

4.16 Did monkeys display different amounts of behaviours in Experiment 1 compared to Experiment 2?

There was a significant difference between affiliative behaviours displayed, self-directed behaviours and maintenance behaviours (Table 4.14, Fig. 4.16). There was however, no significant difference between aggressive- and fear behaviours when females had been presented with the macaque or vet stimuli (Table 4.14). Females displayed more affiliative and self-directed behaviours after seeing the macaque stimuli compared to the vet stimuli. They displayed significantly more maintenance behaviour after presentation of the vet stimuli compared to the macaque stimuli (Fig. 4.16).

Table 4.14. *z*- or *t*-values (d.f.) and *p*-values of a Wilcoxon Signed rank and a Dependent samples *t*-test between behaviours performed after attention bias testing of female rhesus macaques, *Macaca mulatta*, depending on the type of stimuli they were presented with. Significance assigned at p≤0.05*, p≤0.005**, p≤0.001***.

Behaviour	*z*-value/ *t*-value (d.f.)	*p*-value
Aggressive	-0.373	0.709
Affiliative	-2.294	0.022*
Fear	-0.592	0.554

Self-directed	-2.152 (24)	0.042*
Maintenance	3.603 (24)	0.001***

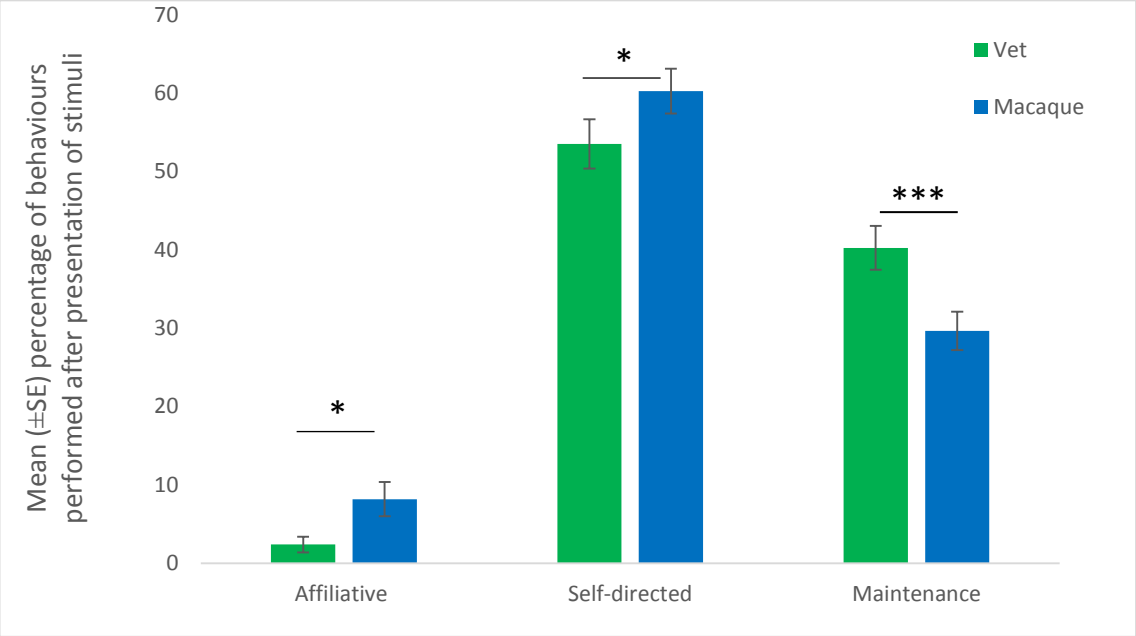

Figure 4.16
Mean (±SE) percentage of behaviours displayed by female rhesus macaques, *Macaca mulatta*, depending on the type of stimuli they had been presented with (vet stimuli n=25, macaque stimuli n=28). Significance was assigned at the p=0.05 level where p≤0.05*, p≤0.005**, p≤0.001***.

Discussion

Impact of genotype on attention bias for vet stimuli (threat vs. non-threat photograph)

HTTLPR and *TPH2* genotype combined had a strong significant impact on attention bias for vet stimuli, where females homozygous for the high-expressing allele in both genotypes were more avoidant of the threat stimuli. Side had a significant impact, where threat stimuli presented to the females' left hand side, received less attention compared to threat stimuli were presented to their right. The attention bias towards the vet stimuli was also influenced by an interaction between age and side. Individuals between 2.5-7.5 and 8.5-9.4 years of age were more vigilant for threat stimuli compared to females between 11.6-15.7 years. Individuals between 11.6-15.7 years were overall less vigilant for stimuli and did not show such a large difference in between the amount of time they spent looking at the threat stimuli on the right compared to the left.

HTTLPR and *TPH2* did not have an impact on attention bias when investigated as separate genotypes (unlike in Chapter 3). However, when classing those two genotypes as one new combined genotype, there was a strong significant impact. Combination 1 carriers, being *HTTLPR* and *TPH2* ll- and ss-homozygous respectively showed significantly greater avoidance of the threat stimuli compared to combination 2 carriers, who were homozygous for one of the genotypes but heterozygous for the other. There were no animals with combination 3 in this data set (*HTTLPR* and *TPH2* ss- and ll-homozygous respectively). Ferguson *et al.* (2012) found an additive effect of genotypes, where an increase in number of low-expressing genotypes was linked to a more blunted adrenocorticotropin-releasing hormone (ACTH) response, leading to dysregulation of the HPA axis pathway. As shown in several studies, the HPA axis is linked to multiple neuropsychiatric disorders, including depression (Chen *et al.* 2010b; Goenjian *et al.* 2012). Further, Brent *et al.* (2013) also found an additive effect of those two genotypes, where individuals with a low-expressing allele in both genotypes socialised less with other individuals. Interestingly, Watson *et al. (*2015) found *TPH2*, but not *HTTLPR* to have an impact on vigilance in free ranging macaques. They suggested that those two genotypes may exert their effects in different ways, a finding supported by the fact that this study did not find *TPH2* alone to have an impact on vigilance for a threat stimulus that would not occur naturally or within social context. Further, they found the low-expressing s-allele of the *TPH2* genotype to be linked to decreased vigilance overall, whilst in this

study, it was linked to increased vigilance for the vet stimulus. Again, this highlights how genotypes impact on behaviour and cognitive processes differently, depending on the context. Vigilance for the threat stimuli seems to be associated with greater emotional vulnerability due to their link to low-expressing alleles and as seen in Experiment 1.

Similar to the conspecific stimuli, side had a major impact on attention bias. When threat stimuli were presented to the monkeys' left hand side, they were less vigilant for it than when threat stimuli were presented to their right hand side. Left gaze bias (LGB) is linked to the right hemisphere, which is responsible for perceptual processing of facial information (Hamilton & Vermeire 1988; Hauser 1993; Butler *et al.* 2005). The overall finding of laterality lends support to the overall direction of LGB and highlights the need to carefully control for an even distribution of side of stimuli presentation during attention bias testing. Monkeys not only show LGB towards conspecifics but also towards humans (Guo *et al.* 2009). As LGB directs the attention towards the left side, it suggests that viewers are able to detect and recognize biologically relevant information more quickly. The finding that, for conspecific stimuli, they showed greater avoidance when aggressive stimuli were presented on their left, is in line with the finding that they showed greater avoidance of the vet threat stimuli on that side. Further, the fact that macaques looked at the threat stimuli more when they were on their right hand side, suggests that it possibly took them longer to interpret the threat stimuli when they were presented on their right and hence were less able to display avoidance.

One possible explanation for the interaction between age and side might be that older females have a lot more experience with the regular veterinary visits. It is possible they understand that they are not always a target of his actions. If females can learn to associate physical pain with the occurrence of a vet check, they might be able to understand that no physical pain puts them at less risk for such a check. Additionally, they might have learned that increased attention to the vet does not impact on the outcome of whether they personally are 'investigated' by him, hence making hugely increased vigilance for him costly and unnecessary. However, it is unlikely that only females that are over ten years old would be able to make this association. As mentioned in the methodology, age correlated with previous exposure in females. The effect of females looking more at the non-threat compared to the threat stimuli might have been influenced by the fact that they had an increased amount of previous attention bias

testing and that they learnt to avoid the negative stimuli presented during those. However, none of the animals had been presented with the vet stimuli previous to this experiment. If females were able to avoid the threat stimuli based on their experience with other types of stimuli, this would suggest that they were able to infer from one type of stimuli to another, which would be a cognitive skill *per se* (Treichler & Raghanti 2010). Further, younger animals might be more likely to become aroused and excited when they realise that others in their social group do. Older females might be less responsive to emotional contagion whilst younger females are more easily influenced and likely to become aroused when they experience other females doing so. It would be interesting to study each individual's injury history in order to see whether those that are more prone to injury exhibit a larger attention bias towards the vet stimuli.

Impact of genotype on behaviour after presentation of vet stimuli

There were several findings for genotype impacting on behaviour after presentation of the vet stimuli. However, all of those were non-significant. There was a trend for *HTTLPR* ll-homozygous females to display increased aggressive approach behaviours and decreased affiliative approach behaviours.

The non-significant trend of *HTTLPR* directly contradicts the non-significant finding for this genotype X behaviour interaction after presentation of the macaque stimuli. Given its previous connection to aggressive and impulsive behaviour, the s-allele should be the one leading to an increase in aggressive behaviours in this study. However, the different stimuli presented here had different emotional values. Spinelli *et al.* (2012) suggested that ll-homozygous macaques would be less able to cope with a stressor upon first exposure compared to s-allele carriers and that in turn, s-allele carriers would increase their stress response over repeated exposure to stress. Maybe, aggressive conspecific males are a regular occurrence to macaque females living in social groups, whilst the vet is a specific stressor that does not occur at such a regular rate. Analyses of larger sample sets of both stimuli are necessary in order to investigate this.

The other non-significant trend was that *HTTLPR* ll-homozygous animals tended to display less affiliative approach behaviours compared to s-allele carriers. If s-allele carriers perceive stressful situations more strongly and show a higher stress response to them, affiliative behaviour might represent a coping mechanism (Cheney & Seyfarth 2009). There was not much variation in affiliative behaviours within the ll-homozygous group

whilst s-allele carriers showed large variation in their affiliative behaviours. It would be interesting to see whether there are other individual differences within the s-allele carrier group that might explain their amount of affiliative behaviour. It has been shown that s-allele carriers respond better to environmental enrichment and social support (Kaufmann *et al.* 2004; Belsky *et al.* 2009; Beaver & Belsky 2012). Possibly females with a baby or their offspring living in the same social group engaged in increased affiliative behaviour in order to cement family bonds. Hence, increases in affiliative behaviour following stress might be a form of social support in rhesus macaques and, as mentioned previously, availability of conspecifics is imperative in order to exhibit such a coping style, mimicking social support.

Impact of genotype on habituation to the vet

Measuring the impact of genotype on attention bias after individuals received habituation revealed several findings. There was no overall significant difference between genotype classes on attention bias scores post-habituation. *DRD4* however, showed a non-significant trend, where T-allele carriers showed more avoidance of the threat stimuli post-habituation compared to AA-homozygous females. There were non-significant differences in aggressive and maintenance behaviours post-habituation between the control and irregular habituation group and a significant difference between aggressive behaviours of the regular habituation and the control group post-habituation. Animals displayed significantly less vigilance for the threat stimuli as well as less fear behaviours during testing post-habituation. The more regular the habituation, the better animals habituated to the vet.

DRD4 has previously been linked to risk-taking and exploration (Coyne *et al.* 2015). If this is the case, AA-homozygous animals could be more willing to take the risk of approaching the vet during habituation sessions and hence learn quicker that he is not a threat, depending on their genotype. This finding was non-significant in this study but is promising for future investigations in habituation studies in animals.

Post-habituation, the irregular habituation group displayed more aggressive approach behaviours compared to the control group, whilst the opposite trend was found for maintenance behaviours. Both those results were non-significant. This was strengthened by the finding that that the regular habituation group displayed significantly more aggressive approach behaviours compared to the control group post-habituation. Hence,

there was a non-significant increase from control to irregular habituation group, followed by a significant increase from control to regular habituation group in aggressive behaviours. However, this was before Bonferroni correction and could be the result of multiple tests being carried out. This is important to note, as presentation of stimuli seemed to elicit an increase in aggressive responses in animals that had received habituation rather than those that had not. One reason might be a feeling of 'frustration' in animals that started to associate the vet partially with food rewards due to the type of habituation they underwent. Upon presentation of stimuli that lacked the associated food rewards, females might have perceived a frustration-like emotion and let this out in form of aggression towards their conspecifics. Additionally, the stressfulness of a stimulus could be a result of its ambiguity (Bethell *et al.* 2012b). The vet could have represented such an ambiguous stimulus, as not all of his interactions during the 12 weeks of habituation were positive. This ambiguity could be what led to the increase in aggressive behaviours. It is however important to note, that not a single attention bias trial was followed by serious injuries or fights due to aggressive behaviour. The increase in maintenance behaviour could have been a response to the appearance of the vet stimuli without following interactions between the vet and individuals of the group. They were not expecting any food off him and once he was no longer present they simply returned to exhibiting normal behaviours such as foraging or resting. It would be valuable to get a baseline activity budget of those groups to confirm that maintenance behaviours in the control group were in fact representative of normal, baseline expression of this behavioural category.

There was a strong significant difference in attention bias score pre- and post-habituation with animals being less vigilant for the threat stimuli after habituation. In detail, the control group did not show a significant difference, although the average attention bias scores indicated a small reduction in vigilance for the threat stimuli after the 12 week gap. There were significant differences between the pre- and post-habituation attention bias scores for the irregular and the regular habituation groups, with both groups being less vigilant for the threat stimuli post-habituation. This effect was stronger in the regular habituation group than the irregular group. This shows that, whilst groups that received habituation did in fact pay significantly less attention to threat stimuli afterwards, the control group did not show such a significant effect. Further, the strength of the effect showed that the more regular habituation they received, the better animals habituated to

the vet. Hence, habituation to an aversive stimulus was successful in those habituated to it. However, the non-significant decrease in attention towards the stimuli in the control group also highlights the importance of considering overall habituation to stimuli over time and repeated exposures.

Considering the four fear behaviours that were recorded during the presentation (lip smack, fear grin, alarm bark and flee), there was a significant difference in the regular habituation group, which displayed more fear behaviours during presentation of vet stimuli pre- compared to post habituation. Hence, habituation in the regular habituation group seemed to reduce the amount of immediate fear responses during presentation of the veterinarians face. This study did not consider the strength of each of those behaviours, where a flee could be interpreted as a greater display of fear compared to a lip smack and fear grin. Future studies should give different weighting to those behaviours in order to create a better representation of the 'fear score'.

Comparison of social *vs.* vet stimuli

One of the major differences between the conspecific- and vet stimuli was that monkeys tended to look more at the neutral- compared to the aggressive macaque stimuli and they looked more at the threat- compared to the non-threat vet stimuli. This means they generally avoided looking at aggressive macaque faces, whilst they were extremely vigilant for the vet faces. This is likely to be due to the different value of the stimuli. Animals at CFM know every individual veterinarian that comes into the facility and start alarm barking the moment they see any one of them (*pers. obs*.). Hence, they seem to be able to infer from seeing the vet to possible invasive procedures happening. At CFM, a veterinarian comes in once a week in order to check on any animals that might have an injury or need monitoring following one. Additionally, the vet is called if an injured or sick animal requires veterinary attention immediately. Once a year, all animals are sedated for an annual health check. Hence, every animal connects the veterinarians to those procedures and fear responses are elicited at a high rate (*pers. obs*.). However, animals are unlikely to have an understanding of whether they personally will be 'targeted' by the vet and hence are increasingly vigilant towards him or her. According to the Error Management Theory (Haselton & Buss 2000) a 'false positive' error occurs when a belief is thought to be true, when in fact it isn't. If individuals assumed the vet to present a danger to them and he did not turn out to be so, this false positive error did not infer a

high cost to them. However, if they did not assume him to be a threat (a false negative), the cost of being caught and examined is high (Haselton & Nettle 2006). Considering that biases have evolved in order to minimize overall costs (Haselton & Nettle 2006), the bias towards the threat stimulus might not be an evolutionary one, but one that is linked to physical pain and injury in macaques that had previous experiences with the vet directly. This was supported by the occurrence of the recorded fear behaviours during presentation of vet stimuli. Whilst conspecific stimuli rarely elicited a strong behavioural response except the 'flee' behaviour, nearly all tested females showed instant fear responses to the threat stimuli. Additionally, if animals elicited an alarm bark, other individuals of the social group immediately increased their vigilance, following gaze direction of the tested female in order to identify the reason for her alarm bark (*pers. obs*.). Hence, the vet is a stimulus that is important to be vigilant for, rather than avoid. Vigilance for the vet allows the female to flee from him, which she could not if she avoided looking at him.

Lastly, when comparing the two stimuli types against each other, females displayed more affiliative approach as well as self-directed and anxiety related behaviours after presentation of the macaque stimuli compared to the vet stimuli. At the same time, they displayed significantly more maintenance behaviours after presentation of the vet stimuli compared to the macaque stimuli. As previously mentioned, affiliative approach behaviours could have represented a type of coping and females reassuring each other, cementing their bonds (Massen & Sterck 2013; Puga-Gonzalez, Hoscheid & Hemelrijk 2015). Further, grooming strengthens friendships and it is not unusual for a group of macaque females to display extreme aggression towards a new male (*pers.obs*., Chapais 1991). If such aggression of females towards a new male is occurring, it is based on a strong connection between those females in order to ensure unity against a male that is only outcompeted by a set of cooperating females. As a breeding facility, CFM does occasionally swap males between social groups in order to ensure genetic variation and avoid inbreeding. Hence, females have possible past experiences with new males joining their social groups. Contrarily, females have no opportunity to act as a team against the vet when he appears. Hence, affiliative behaviour between them did not need to cement bonds that were not needed.

The increase in self-directed and anxiety related behaviours could have to do with the learned effect of the vet's leaving meaning that no further interaction with him will follow. The presence of an unknown aggressive male conspecific however is new and females may have had no idea of the type of consequences his appearance would have, creating uncertainty and possibly increasing anxiety in those animals.

Considering that during both studies, aggressive behaviour seemed to increase following stimuli presentation, it is not surprising that there was no significant difference in its amount between the stimuli types. Fear-avoid behaviours were not significantly affected by any of the genotypes or presentation of the stimuli and were some of the hardest to code, as often submissive behaviour is subtle and easy to miss by the observer. Lip smacks or fear grins might also have been missed if the animal had its' back fully or partially turned towards the observer. Maintenance behaviours could have been significantly more following presentation of the vet stimuli due to an unplanned and uncontrolled for increase in regular feedings. It is however also possible that interaction with their infants was increased in females following presentation of the vet stimuli which could be a type of substitute to grooming behaviour with other conspecifics.

Chapter 5

This study aimed to identify the impact of genotype on attention bias in rhesus macaques. This feeds into a large project to identify a novel way of measuring welfare of captive macaques as well as, hopefully, increasing welfare. The research conducted in this study provided insight into the impact of genotype on attention bias of rhesus macaques towards two different stimuli sets, as well as the effect on behaviour following experiments. The general aim of this study was met: genotype does show an impact on individual's attention bias.

Discussion

As shown in this study, genotype has an impact on attention bias in rhesus macaques, but this impact seems to be specific to the presented stimuli. Socially relevant stimuli elicited a response which was impacted by an interaction between the nursing status and side of presentation, as well as the *HTTLPR* and *MAOA* genotypes. A human aversive stimulus was affected by the age of the individual in interaction with the side of presentation, as well as a combination of the *HTTLPR* and *TPH2* genotype.

Studies of impact of attention bias in humans have found somewhat opposite effects to those found in this study. Looking time tasks generally assume that the longer an individual looks at a stimulus the more interesting it is to it (Winters, Dubuc & Higham 2015). That might be wrong, however, in regards of primates in which direct stare is a threat (Machado & Bachevalier 2008). If an animal is presented with a conspecific's face, then looking at it for a prolonged amount of time confers the risk of 'challenging' this individual. Bethell *et al.* (2012b) have shown that male macaques are even less likely to take such a risk after they have been through a health check, possibly because they were already in an elevated state of stress and did not want to engage in such potentially dangerous competition. Hence, the results achieved here do not present a fundamental difference to the impact of genotype on attention bias between humans and rhesus macaques. Rather, they are in line with our knowledge of rhesus macaque behaviour.

DRD4, *HTTLPR* and *MAOA* genotypes impact on aggressive, affiliative and maintenance behaviours in different ways but some results here were non-significant and need further tests to be confirmed. Further, *DRD4* genotype might have an effect on the success of

habituation, where T-allele carriers showed a non-significant increase in avoidance of threat stimuli after regular habituation.

Ferguson *et al.* (2012) found an additive effect of risk genotypes in rhesus macaques, as did other studies (Brent *et al.* 2013; Armbruster *et al.* 2009). The research presented here did also find such an effect. However, the studied individuals only presented two out of three possible combinations of the *HTTLPR* and the *TPH2* genotype. A larger data set including the third combination might give further insight into the impact of genes. It has been suggested that high-expressing alleles of one genotype might be able to buffer an individual from adverse effects of low-expressing allele in another genotype (Dettmer & Suomi 2014). If this is the case, combination 2 carriers would possibly have more of a buffer compared to combination 3 carriers and there should be an increase in effect from one combination to the next.

As mentioned in the introduction, some of the alleles studied here have been labelled 'risk' alleles. This would imply that those alleles only bear disadvantages to their carriers and this would mean that, due to survival of the fittest (Darwin 1869), those alleles should be extinct in those species in which they bring disadvantages. Yet, some genotypes, like the *HTTLPR* s-allele have been shown to be linked to increased cognitive functioning and flexibility, responsiveness to social support and creative dancing (Roiser *et al*. 2007; Finger *et al.* 2007; Taylor *et al*. 2006; Bachner-Melman *et al.* 2005). Recently, *HTTLPR* and *TPH2* low-expressing alleles have been linked to increased environmental exploration and vigilance in macaques (Spinnelli *et al.* 2012; Watson *et al.* 2015). Dobson & Brent (2013) suggested that *HTTLPR* ll-homozygous primates would fare better in a more stable environment where competition levels don't differ substantially from the norm, whilst s-allele carriers would be able to adapt better when intra-group competition levels are higher than usual. The same could be the case for *MAOA* 7-repeat allele carriers and non-carriers. Environments are ever changing and increased vigilance would be an advantage in an unstable environment where food resources are low or many predators are present. However, in a stable environment it would be a waste of energy. Hence genetic variation is thought to be selected for by variable environments and each genotype bringing advantages as well as disadvantages depending on the current environment (Suomi 2006). Rhesus macaques have been very successful in colonising new

habitats, possibly because they have the genetic flexibility to adapt behaviourally to new environments (Charkraborty *et al.* 2010).

Most studies on impact of genotype on behaviour in macaques have compared normally reared monkeys against those that were reared in a nursery with only same aged peers or by hand. Those studies, in line with those on humans, found a strong interaction between early life experience and genotype (Caspi *et al.* 2003; Barr *et al.* 2004a, b, c; Newman *et al*. 2005; Kinnally *et al.* 2008, 2010). However, not many studies have investigated the impact of genotype between groups of normally reared macaques (Spinelli *et al.* 2012; Sullivan, Mendoza & Capitanio 2011). This study hence not only was the first to investigate the impact of genotype on attention bias, but also added to our understanding of impact of genotype on behaviour under 'normal' rearing and housing conditions of captive macaques.

In Guo *et al*.'s (2009) study on laterality of gaze in rhesus macaques, they used head restraints, eye coils and monkey chairs. Those are invasive methods, raising ethical concerns as well as only allowing study of a small sample size. This study identified monkeys gaze through non-invasive positive reinforcement methods during tests in which females took part voluntarily, allowing for a large number of individuals to be tested. Thatcher (2015) shows that, although more time intensive, training monkeys to station in order to make them perform tests is possible, less invasive and more rewarding for humans and animals involved. This shows that such invasive methods are unnecessary and researchers wanting to investigate things such as gaze patterns of monkeys should invest the time to train their study subjects through positive reinforcement to allow testing under non-invasive, more ethical conditions.

The methods used in this study had advantages and disadvantages. A possible disadvantage is that this study only tested animals that took part voluntarily. Although other females than those tested here were trained to sit by their station (Thatcher 2015), they were too nervous to remain there for a sufficient amount of time to present them to the attention bias paradigm. Often, those animals that would not take part were low-ranking. This means that individuals that are most sensitive and stress avoidant were not included in this study as well as other studies investigating the effect of genotype on cognition (Homberg & van den Hove 2012). However, animals that are trained through positive reinforcement techniques and that take part in tests voluntarily also improve the

quality of findings obtained in those studies (Rennie & Buchanan-Smith 2006). Further, this study only tested female rhesus macaques, hence lacking data of genotype on attention bias in males. However, females are more prone to depression and female macaques have greater HPA axis responses to stressors than males of the same genotype (Barr *et al.* 2004d), therefore putting them at greater risk of suffering from psychological distress in captivity. Another disadvantage was the fact that the whole social group was stationed during testing, meaning that individuals that had not been tested yet could have already seen the stimuli whilst one of their conspecifics was tested. However, this predictability of the upcoming stimuli was somewhat decreased by the fact that all animals were presented with the test stimuli, followed by either fruit or conspecific infant stimuli. Hence, there was still some unpredictability for the stimuli when they were revealed. Lastly, rank was not assessed for this study. An objective measure of each individuals' rank would allow for this to be included in the statistical analysis.

This study found a surprising effect of nursing status in interaction with the side on which the stimuli were presented to the female and I argue that this was due to human error in the planning of the tests as those females were mostly stationed in a particular part of the enclosure. This shows that AB testing is only reliable when other factors are strongly controlled for. It is necessary to take into consideration things such as the location of testing to avoid wrong interpretation of results.

Various things to consider during looking time paradigm testing were discussed in a review by Winters, Dubuc & Higham (2015) and met in the methodology of this study. Video recording of trials allowed for subsequent coding, improving the precision of measurements as well as allowing calculation of inter-observer reliability. Further, all aspects of the visual scene were standardized and the stimulus type as well as orientation of the head was counterbalanced in all stimuli. Stimuli pairs were further matched in colour and luminance, the order of presentation was randomized and counterbalanced, the apparatus was placed similarly in all trials and the simultaneously presented stimuli were presented at the same distance from the individual's position. Further, researchers were blind to the condition of the current trial as well as during coding of the videos.

Possibly the greatest advantage of attention bias testing, is that underlying emotion-states can be detected which would not be measurable by behavioural observations (Bethell 2015). Other physiological measures can identify a state of arousal in individuals,

but are unable to distinguish whether this is due to a positive or negative (i.e. food or aggression) underlying cause (Mendl, Burman & Paul 2010; Hemsworth *et al.* 2015). Knowing an individual's genotype can aid in explaning variation in studied groups as well as identify those that are more prone to anxiety or stress due to their genetic make-up. Even when genotyping is not possible, being aware of its impact allows for a more informed interpretation of attention bias data to be made. This allows individual assessment of animals and, if carried out over time, can detect changes in emotion, and hence wellbeing, over time (Bethell 2015).

Future research

Kinnally *et al.* (2010) suggested that macaques carrying a combination of *HTTLPR* and *MAOA* genotypes, where either one or both are present in their low-expressing form, would exhibit the least behavioural inhibition in response to a stressful situation due to their increased impulsiveness. Further, the presence of high-activity genotypes might buffer individuals from presence of low-activity ones in other genes (Kinnally *et al.* 2010), hence making it harder to detect an impact of one genotype alone. Analysis of the results presented here with the *HTTLPR* and *MAOA* genotype did not show an interaction between the two to impact on attention bias, whilst *HTTLPR* and *TPH2* did. Watson *et al.* (2015) also found that males were more vigilant than females. Maybe, in a study that investigated the impact of genotype on attention bias in male rhesus macaques, there would be different findings. A larger number of individuals is needed in order to increase the availability of different genotypes and the possible combinations between those. This will enable testing of previous findings such as the interaction of *TPH2* and *HTTLPR* shown here and elsewhere (Brent *et al.* 2013) as well as an interaction between *MAOA*, *HTTLPR* and *TPH2* genotypes (Kinnally *et al*. 2010). Future studies should investigate several possible combinations of genotypes in individuals, maybe combining even more than two in order to see whether there are combinations that put an individual at high risk. For example, it is likely that *HTTLPR*, *TPH2* and *MAOA* genotypes interact, as all three are implicated in the serotonin pathway.

The *OPRM1* genotype, which did not show impact on any of the investigated factors in this study, has been linked to attachment between mother and infant. Future stimuli could test stimuli sets of known *vs*. unknown or unknown *vs*. own infants in females to investigate whether *OPRM1* impacts on an attentional bias towards infants. The identified

DRD4 SNP needs further investigation in terms of its' function *in vitro*, as well as additional sequencing to investigate whether this SNP was in fact the reason for significant effects found in this study or simply in linkage disequilibrium with a causal SNP.

In terms of testing conditions, the position of individuals within the testing area should be controlled for, depending on the type of environment, to avoid confounding effects due to repeated testing in one position. Further, individuals that will be tested in one session should be spaced apart in a way that does not allow them to see the stimuli before their turn of testing, whilst still allowing for the social group to be present during testing as to not create an unfamiliar, stressful environment that is not 'natural' to the tested individual (i.e. isolation from the group). It may also be possible that the apparatus could be modified in a way that includes blinds, restricting the view onto the stimuli from any position that is not right in front of it.

Eye tracking software is currently being developed (E. Bethell, *pers. comm*.) which would allow for precise coding of eye gaze, possibly decreasing human error and the amount of time and effort needed currently for manual coding of videos. Additionally, the behavioural categories recorded for this study were pooled into five distinctive categories. It would be interesting to investigate some of those categories, such as vigilance, threat, or grooming, separately in relation to genotypes as this might reveal new findings of genotypes on those specific behaviours.

Conclusion

This study was the first to show the impact of genotype on attention bias in female rhesus macaques. As attention bias is developed as a tool to investigate well-being and emotion-state of captive primates, these findings should be considered in regards to females' at higher risk to perceive stress compared to conspecifics with genotypes that act as a buffer against such stress. Further, knowing and understanding the way laterality impacts on attention bias is crucial in order to interpret findings as well as the development of methods.

Due to the increase of aggressive behaviour following attention bias testing in this study, general testing should be spaced out considerably and individuals should be observed closely following testing. Baseline data of activity budgets should be collected in order to

investigate whether attention bias testing leads to changes in behaviour short- or long-term.

Breeding captive populations in order to achieve that all animals carry only those genotypes that makes them less prone to suffer would not be a solution. Especially in biomedical research it is important to obtain a healthy population which represents the closest possible model to that found in the wild. In order to investigate possible side effects of medication for example, a sewed genepool would be a great disadvantage. Instead, if animals are known to carry genotypes that put them at greater risk of suffering, measures to assure a high standard of welfare should be increased. Even if an insitiution is not able to genotype their animals, they have to be aware about the range of possible variation between animals. Studies investigating the impact of genotype on behaviours add to our knowledge as to how big this variation can be.

Measuring cognitive bias provides an objective measure of emotion in animals. If individuals are presented with cognitive bias tasks repeatedly over time, it can establish a baseline profile for the individual. If changes of the baseline measurements towards a more negative bias are detected, and are consistent over a period of time, the individual can be identified as being at risk of suffering and decreased welfare. In individuals that are found to be of a negative, depressive-like inner state, attention bias testing could then help to further identify the source of stress by presenting specific stimuli to the individual and identifying which one elicits a large response. If habituation to adverse stimuli is possible, then it is important to ensure it is carried out regularly enough for all individuals, especially those that might need more habituation in order to achieve habituation. After habituation, attention bias can also be used in order to evaluate whether it was successful or not, and to what extent.

It is imperative to develop methods to assess cognitive and attention biases as far as possible to allow easy, fast and cheap replication for any person working with captive animals. This will allow for those methods to be applied readily and give way for appropriate interventions to improve well-being as early as possible in order to provide those animals that are within our care with the best welfare humanly possible.

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Appendix 1:

Agarose gels

a) 1.5% NuSeive agarose gel (Thermo Scientific, TopVision agarose 100g) was prepared by mixing 100ml of 1xTBE buffer with 1.5g of agarose and microwaving it until no particles remained. The liquid was cooled down to room temperature and 5µl of red die (Biotium, GelRed™ Nucleic Acid Gel Stain, 10.000x in DMSO) were added before pouring the liquid into a 10x15cm tank with either one or two combs with 20 wells each, in which PCR products were pipetted for electrophoresis.

b) 3.5% NuSeive agarose gel (Thermo Scientific, TopVision agarose 100g) was prepared by mixing 70ml of 1xTBE buffer with 2.45g of agarose, microwaving it until no particles remained and adding 5µl of gel red (Biotium, GelRed™ Nucleic Acid Gel Stain, 10.000x in DMSO) after cooling it to room temperature. The liquid was then poured in a 7x15cm tank with one comb with 20 wells, into which PCR products were pipetted for electrophoresis.

PCR product Purification

50µl of successful PCR mix was mixed with 50µl of binding buffer in a PCR tube (Alpha Labs, 2ml Thin Wall Tube Flat Cap) and the mix was then pipetted into the purification column (provided with the GeneJet PCR product purification kit). Columns were then centrifuged for one minute, the collection tube was taken off and the liquid in it discarded. The wash buffer of the purification kit was diluted with 45ml of 100% ethanol. 700µl of the wash buffer was then added to the column and centrifuged for one minute before the flow through in the collection tube was discarded. An additional 1min centrifugation was undertaken to ensure removal of all ethanol and the flow through was discarded. The column was then placed in a clean 1.5ml Eppendorf tube and 30µl of provided elution buffer were added directly onto the column matrix, without breaking the membrane. The tube was left to rest for one minute and then centrifuged for one minute. After centrifuging, the column was taken off and the purified DNA product was left in the bottom of the Eppendorf tube. For sequencing purposes, 5µl of the purified DNA was added to 5µl of the forward primer (at 5pmol/µl) in a 1.5ml Eppendorf tube. Those were labelled with barcodes and sent off for sequencing.

Appendix 2:

List of genotypes of all 65 female rhesus macaques, *Macaca mulatta*, analysed for this study.

Animal	Genotype								
	TPH2	MAOA	OPRM1	HTTLPR	DRD4, 447934	DRD4, 448009	DRD4, 448055	DRD4, 448080	DRD4, 448097
Dime	SS	5 7	GG	SS	GG	AT	GG	GG	GG
Dolly	SL	5 5	CC	LL	GG	AA	GG	GG	GG
Green	SL	7 7	CG	SL	GG	AA	GG	GG	GG
Hatty	SS	7 7	CC	SL		TT	GG	GG	GG
Hazel	SS	5 5	CC	LL	GG	AT	GG	GG	GG
Helga	SL	5 7	CC	LL	GG	TT	GG	GG	GG
Hetty	SS	6 7	CC	LL	GG	AA	GG	GG	GG
Hillary	SS	7 7	CC	SL	GG	AT	GG	GG	GG
Hilda	SS	6 7	CC	LL		AA	GG	GG	GG
Holly	SS	5 5	CG	LL	GG	TT	GG	GG	GG
Hope	SL	6 7	CC	LL		TT	GG	GG	GG
Lala	SS	7 7	CC	LL	GG	AT	GG	GG	GG
Leah	SL	5 7	CC	SL	GG	AT	GG	GG	GG
Libby	SL	5 7	CC	SL	GG	AT	GG	GG	GG
Love	SS	7 7	CC	SL	GG	AA	GG	GG	GG
Lydia	SL	5 7	CC	LL	GG	TT	GG	GG	GG
Meesha	SS	5 5	CC	LL	GG	AA	GG	GG	GG
Meg	LL	5 5	CC	LL	GG	AA	GG	GG	GG
Melody	LL	5 7	CC	LL		AA	GG	GG	GG
Ocelot	SS	6 6	CC	SL	GG	AT	GG	GG	GG
Paca	SS	7 7	CC	LL		TT	GG	GG	GG

Animal	Genotype								
	TPH2	MAOA	OPRM1	HTTLPR	DRD4, 447934	DRD4, 448009	DRD4, 448055	DRD4, 448080	DRD4, 448097
Pamela	SS	6 7	CG	LL	GG	TT	GG	GG	GG
Pandora	SS	5 7	CC	SL	GG	AT	GG	GG	GG
Patricia	SS	6 7	CC	SL	GG	AT	CG	GG	GG
Pax	SL	5 5	CC	LL	GG	AA	GG	GG	GG
Pidray	SS	7 7	CC	LL	GG	AT	CG	GG	GG
Polka	SS	5 7	CC	LL	GG	TT	GG	GG	GG
Porsche	SL	6 6	CC	LL	GT	AT	GG	GG	GG
Shirley	SS	6 7	CC	SL	GG	TT	GG	GG	GG
Spangle	SS	6 7	CC	SL		AT	GG	GG	GG
Tass	SS	6 6	CC	SS	GG	AT	GG	GG	GG
Tes	SS	5 6	CC	LL	GG	AA	GG	GG	GG
Venice	SS	5 7	CG	SL		AT	GG	GG	GG
Doreen	SL	7 7	CC	SL	GG	AT	CG	CG	AG
Girl	SL	5 7	GG	SL		AA	GG	GG	GG
Lake	SS	6 6	CC	SL	GG	AT	CG	CG	AG
Mustard	SS	6 7	CC	SL	GG	AA	CC	GG	GG
Omelette	SS	7 7	CG	SL	GG	AT	GG	GG	GG
Orlanda	SS	7 7	CC	SS	GG	AA	GG	GG	GG
Pansy	SS	5 7	CG	SL	GG	AT	GG	GG	GG
Penny	SS	6 7	CC	LL	GG	TT	GG	CG	AG
Senga	SS	5 7	CC	SL	GG	AT	GG	GG	GG
Simone	SL	6 7	CC	SL	TT	AA	GG	GG	GG
Sizzle	SS		CC	SL	GG	AT	GG	GG	GG

Animal	Genotype								
	TPH2	MAOA	OPRM1	HTTLPR	DRD4, 447934	DRD4, 448009	DRD4, 448055	DRD4, 448080	DRD4, 448097
Tanya	SS	6 6	CC	SL	GG	AT	GG	GG	GG
Thistle	SL	7 7	CC	LL	GG	AT	GG	GG	GG
Tulip	SS	5 7	CC	SL	GG	TT	GG	GG	GG
Uno	SS	6 7	CG	LL	GG	TT	GG	GG	GG
Vienna	SS	6 7	CC	LL	GG	AT	CG	GG	GG
Kandy	LL	6 7	CC	LL	GG	AT	GG	CG	AG
Kelly	SL	6 7	CG	LL	GG	AT	GG	GG	GG
Kit	SL	5 6	CC	LL	GG	AT	CG	CG	AG
Linz	SS	5 6	CC	SL	GG	AT	CG	CG	AG
Maj	SS	5 7	CC	SL	GG	AA	GG	GG	GG
Maureen	SS	5 7	CC	SL	GG	AA	GG	GG	GG
Orinoco	SS	7 7	CG	LL	GG	AT	GG	GG	GG
Razz	SL	5 7	CG	LL	GG	AT	GG	GG	GG
Rene	SS	5 7	CC	SL	GG	AA	GG	GG	GG
Rhumba	SS	5 7	CC	SL	GG	AT	GG	GG	GG
Robyn	SL	6 7	CC	SL		TT	GG	GG	GG
Ruby	SS	6 6	CG	SL	GG	AT	GG	GG	GG
Shallot	SS	5 7	CC	SL	GG	AT	GG	GG	GG
Venus	SS	6 6	CC	LL	GG	AT	GG	CG	AG
Verity	SS	6 7	CC	LL	GG	AA	GG	GG	GG
Wasabi	SS	6 7	CC	SL	TT	AA	CG	GG	GG

Appendix 3:

Matrilines (as far as available) of all female rhesus macaques, *Macaca mulatta*, that took part in the attention bias Experiment 1: unknown conspecific faces (aggressive – neutral)

F265			
	Honey		
		Sizzle	
		Tes	
	Hannah1		
		Ocelot	
			Tass
		Shirley	
	Arif		
		Lydia	

Camilla			
	Zoe		
		Dora	
			Libby

A76		
	Helga	
		Porsche
		Tanya

A9		
	186	
		Hetty

M26		
	Mustard	
		Vienna
		Wasabi

Eileen		
	156	
		Orlanda

258				
	A26			
		Astrid		
			Leah	
			Rene	
			Shallot	
		Charlotte		
			Hatty	
				Spangle
			Omelette	
			Ruby	

152	
	Lala

Ann	
	Hope
	Melody
	Robyn

Oslyn	
	Rhumba

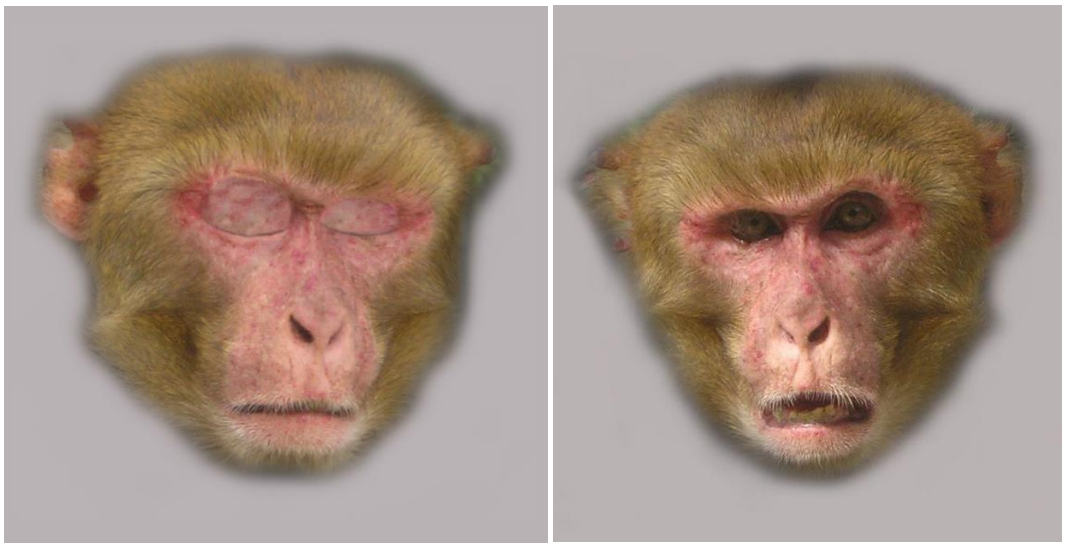
Alice	
	Holly
	Razz

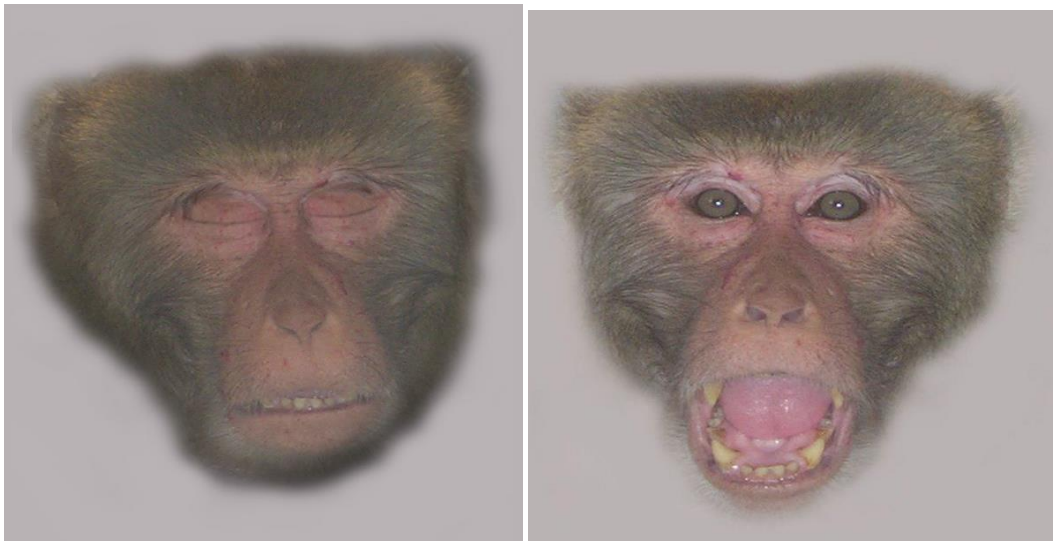
Phylis			
	Audrey		
		Abbey	
			Hazel
			Meesha

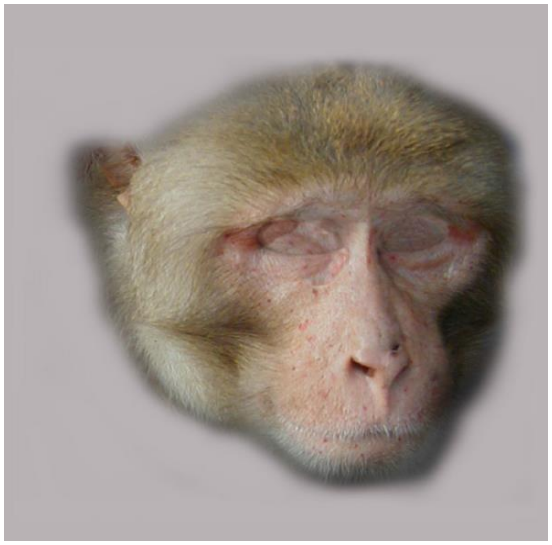
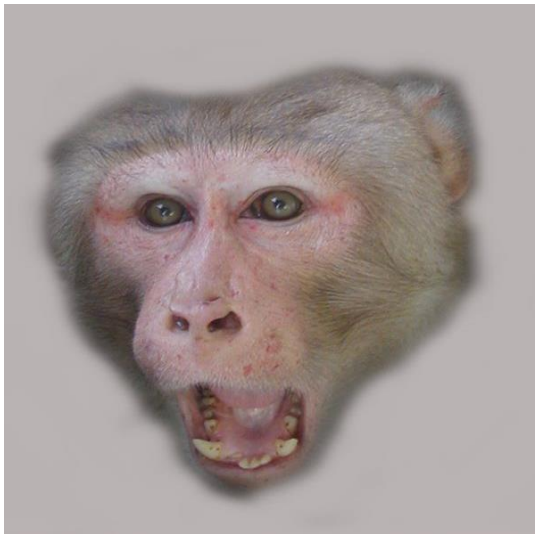
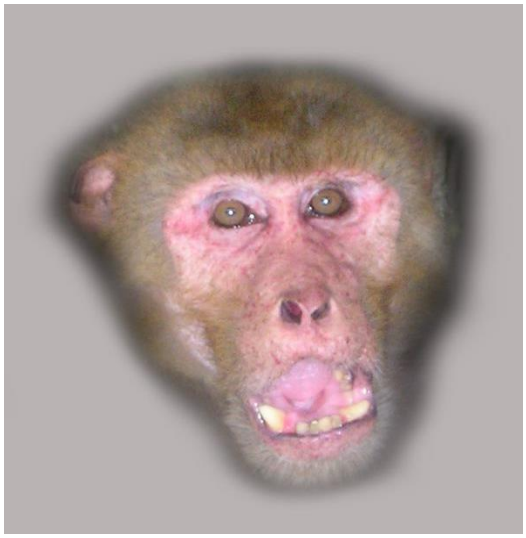
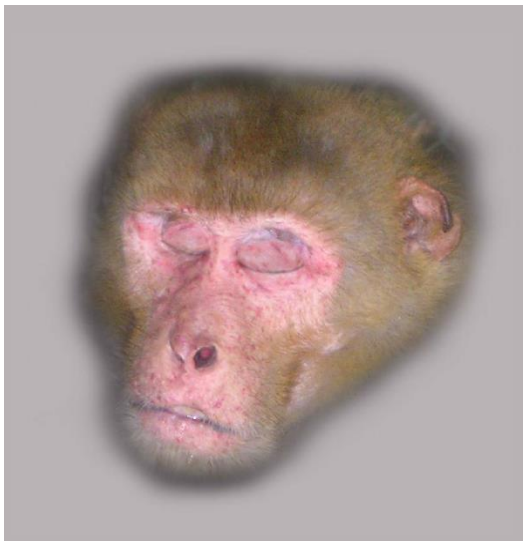
Irene			
	Saphire		
		Hilda	

Appendix 4:

The four stimulus pairs of the unknown conspecific males (aggressive – neutral). Each stimulus was presented to the left or right as decided by pseudo randomization. Stimuli were mirrored so they faced inwards on every presentation. Photographs courtesy of E. Bethell and used with her consent.





Appendix 5:

30 Candidate models

1<-glmer(AB ~ AgeMos + Group + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
2<-glmer(AB ~ AgeMos + NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
3<-glmer(AB ~ AgeMos + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
4<-glmer(AB ~ AgeMos + Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
5<-glmer(AB ~ Group + NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
6<-glmer(AB ~ Group + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
7<-glmer(AB ~ Group + Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
8<-glmer(AB ~ NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
9<-glmer(AB ~ NursStat + Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
10<-glmer(AB ~ AgeMos* Group + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
11<-glmer(AB ~ AgeMos*NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
12<-glmer(AB ~ AgeMos* Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
13<-glmer(AB ~ Group *NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
14<-glmer(AB ~ Group * Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
15<-glmer(AB ~ NursStat* Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
16<-glmer(AB ~ AgeMos* Group + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
17<-glmer(AB ~ Side + AgeMos* Group + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
18<-glmer(AB ~ NursStat + AgeMos* Group + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
19<-glmer(AB ~ Group + AgeMos* Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
20<-glmer(AB ~ Side + AgeMos*NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
21<-glmer(AB ~ Group + AgeMos*NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
22<-glmer(AB ~ NursStat + AgeMos* Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
23<-glmer(AB ~ Group + AgeMos* Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
24<-glmer(AB ~ AgeMos + Group *NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
25<-glmer(AB ~ Side + Group *NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
26<-glmer(AB ~ AgeMos + Group * Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)

```
27<-glmer(AB ~ NursStat + Group * Side + (1|Presentation)  + (1|Matriline/MonkeyID), data = dat, family=binomial)
28<-glmer(AB ~ AgeMos + NursStat* Side + (1|Presentation)  + (1|Matriline/MonkeyID), data = dat, family=binomial)
29<-glmer(AB ~ Group + NursStat* Side + (1|Presentation)  + (1|Matriline/MonkeyID), data = dat, family=binomial)
30<-glmer(AB ~ NursStat + Group + Side + (1|Presentation)  + (1|Matriline/MonkeyID), data = dat, family=binomial)
```

Appendix 6:

Table 5.1. Normality distribution of behavioural categories for the aggressive – neutral conspecific macaque faces as assessed by running a Shapiro-Wilk test. Significance assigned at $p \leq 0.05$*, $p \leq 0.005$**, $p \leq 0.001$***.

Behavioural category	Median (Q1, Q3)	*p*-value
Aggressive approach	1724 (0, 6475.5)	$p<0.001$***
Affiliative approach	34986 (0, 184607.5)	$p<0.001$***
Fear avoid	0 (0, 3106)	$p<0.001$***
Self-directed and anxiety	717426.5 (609873.5, 875916.75)	$p=0.565$
Maintenance	381238 (243719.5, 507434.5)	$p=0.267$

Appendix 7:

Normal distribution of the best fit model of aggressive- neutral conspecific faces.

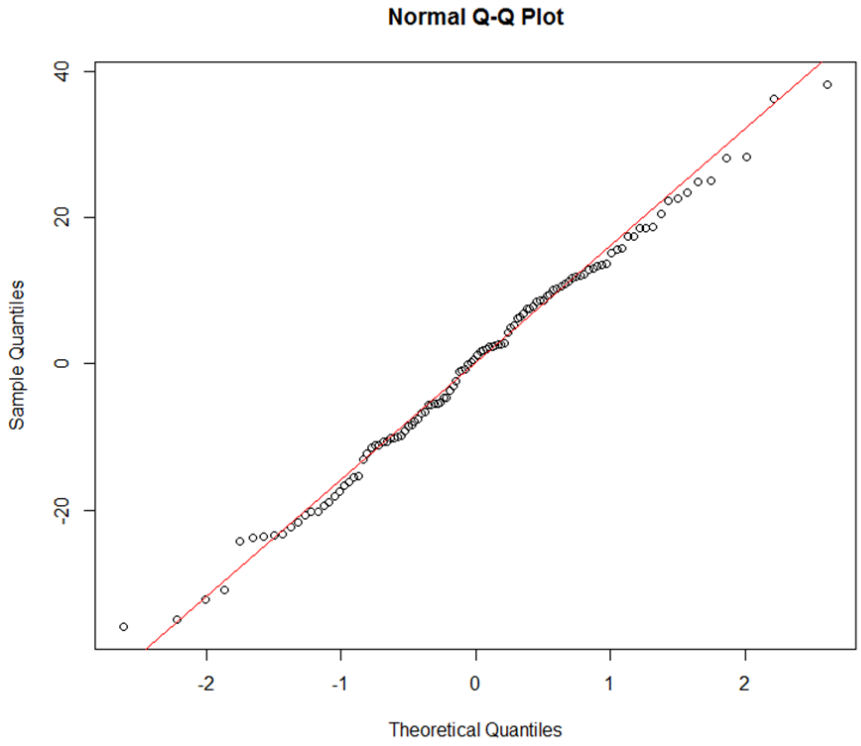


Figure 6.1
Quantile-quantile plot of the best fit model to explain attention bias data of 28 female rhesus macaques, *Macaca mulatta*, including an interaction of side and nursing status as well as *HTTLPR* and *MAOA*. Line shows line of best fit.

Appendix 8:

Matrilines (as far as available) of all female rhesus macaques, *Macaca mulatta*, that took part in the attention bias Experiment 2: veterinarian (threat – non-threat)

F265			
	Honey		
		Sizzle	
		Tes	
	Hannah1		
		Ocelot	
			Tass
		Shirley	

Phylis			
	Audrey		
		Abbey	
			Hazel
			Meesha

Irene			
	Saphire		
		Hilda	

A76		
	Helga	
		Porsche
		Tanya

M26		
	Mustard	
		Vienna
		Wasabi

Ann	
	Hope
	Melody
	Robyn

Alice	
	Holly
	Razz

152	
	Lala

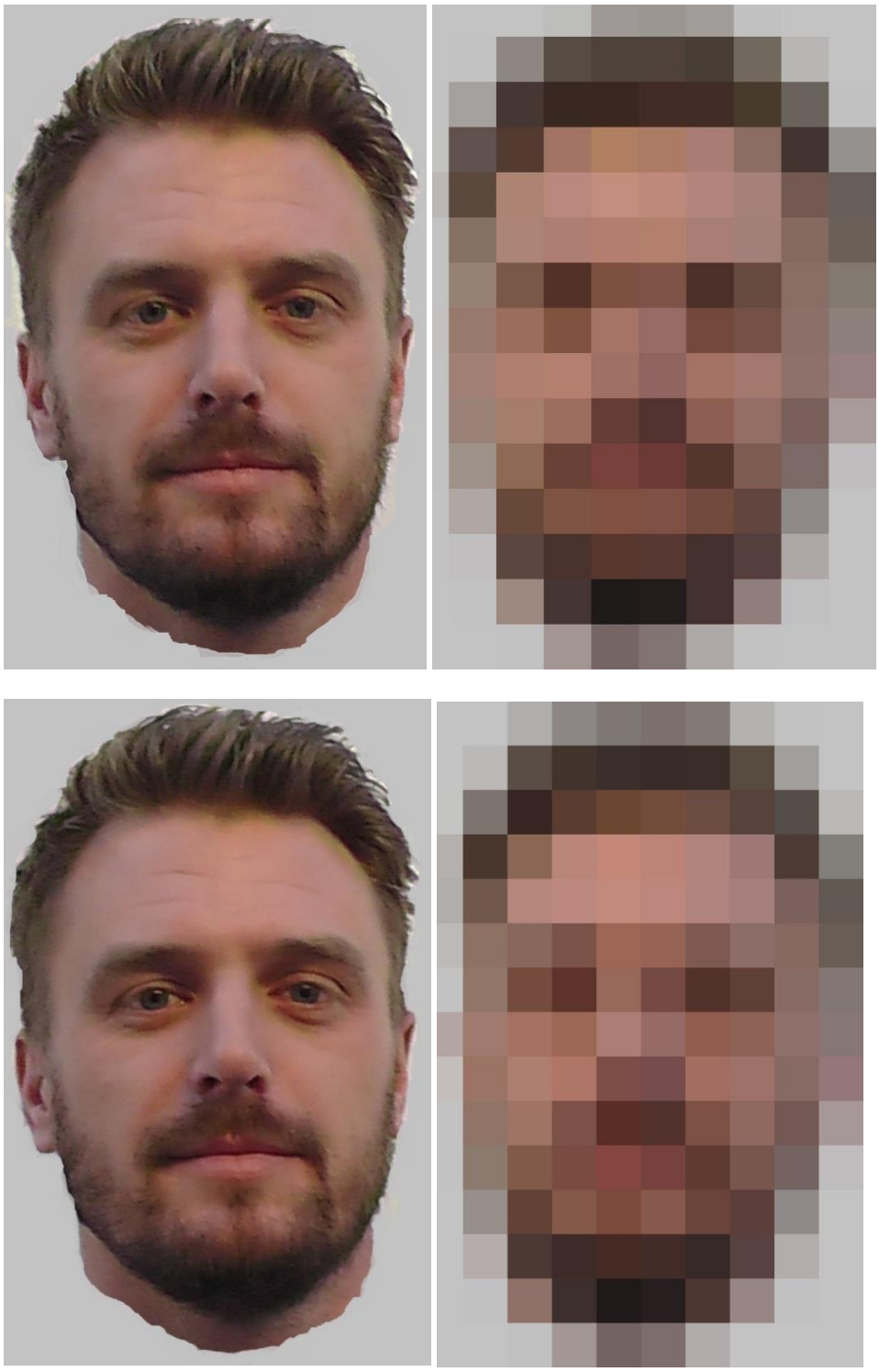
Oslyn	
	Rhumba

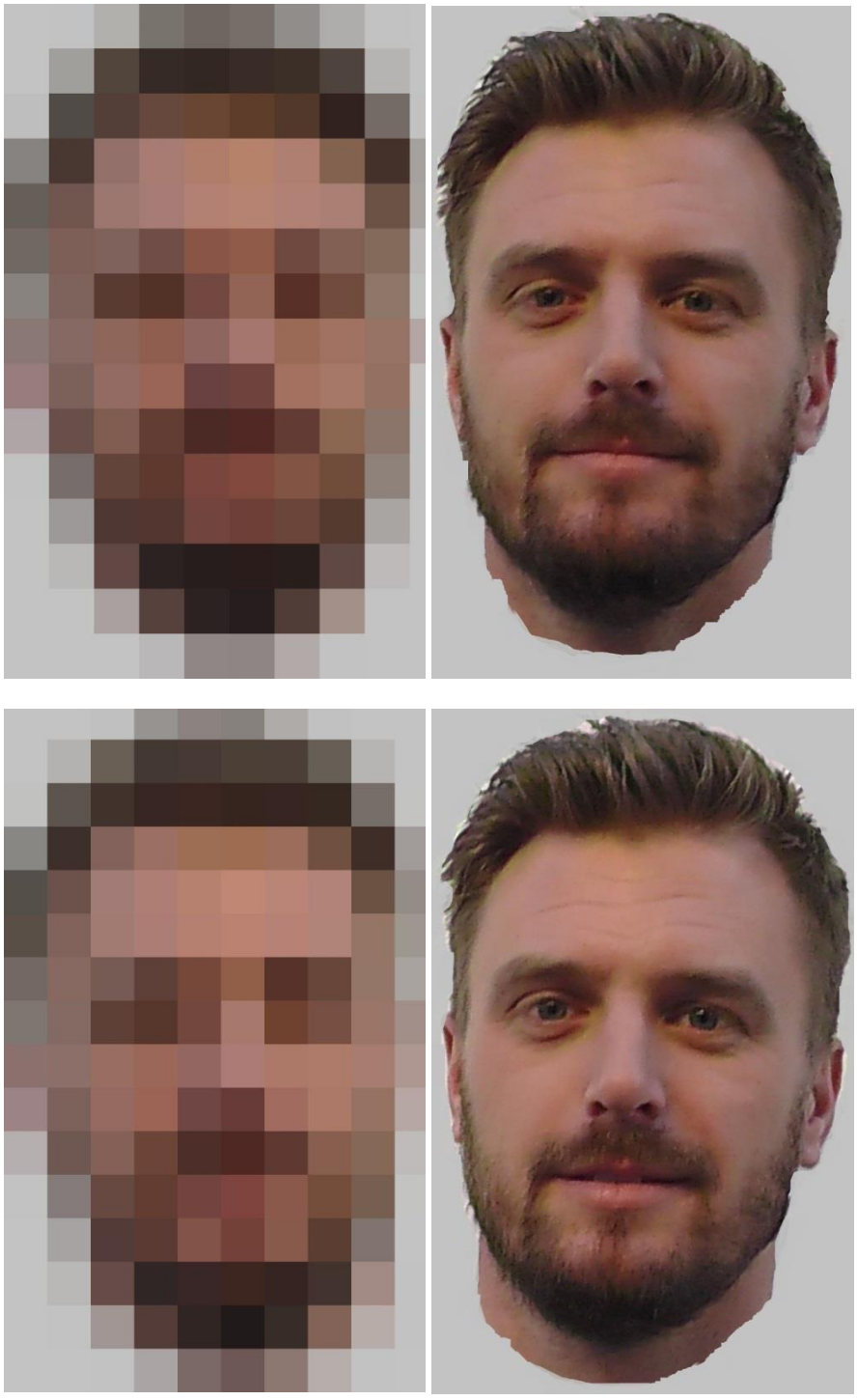
Camilla			
	Zoe		
		Dora	
			Libby

258				
	A26			
		Astrid		
			Leah	
			Rene	
			Shallot	
		Charlotte		
			Hatty	
				Spangle

Appendix 9:

The four stimulus pairs of the veterinarian with the matching photograph mirrored and pixelated. Luminance and size matched. Each stimulus was presented to the left or right as decided by pseudo randomization. Stimuli always faced inwards. Photographs used with consent of the veterinarian.





Appendix 10:

30 Candidate models

1<-glmer(AB ~ AgeMos + Group + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
2<-glmer(AB ~ AgeMos + NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
3<-glmer(AB ~ AgeMos + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
4<-glmer(AB ~ AgeMos + Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
5<-glmer(AB ~ Group + NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
6<-glmer(AB ~ Group + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
7<-glmer(AB ~ Ra Group nk + Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
8<-glmer(AB ~ NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
9<-glmer(AB ~ NursStat + Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
10<-glmer(AB ~ AgeMos* Group + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
11<-glmer(AB ~ AgeMos*NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
12<-glmer(AB ~ AgeMos*Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
13<-glmer(AB ~ Group *NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
14<-glmer(AB ~ Group *Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
15<-glmer(AB ~ NursStat*Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
16<-glmer(AB ~ AgeMos* Group + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
17<-glmer(AB ~ Side + AgeMos* Group + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
18<-glmer(AB ~ NursStat + AgeMos* Group + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
19<-glmer(AB ~ Group + AgeMos*Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
20<-glmer(AB ~ Side + AgeMos*NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
21<-glmer(AB ~ Group + AgeMos*NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
22<-glmer(AB ~ NursStat + AgeMos*Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
23<-glmer(AB ~ Group + AgeMos*Side + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
24<-glmer(AB ~ AgeMos + Group *NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)
25<-glmer(AB ~ Side + Group *NursStat + (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)

```
26<-glmer(AB ~ AgeMos + Group *Side + (1|Presentation)  + (1|Matriline/MonkeyID), data = dat, family=binomial)
27<-glmer(AB ~ NursStat + Group *Side + (1|Presentation)  + (1|Matriline/MonkeyID), data = dat, family=binomial)
28<-glmer(AB ~ AgeMos + NursStat*Side + (1|Presentation)  + (1|Matriline/MonkeyID), data = dat, family=binomial)
29<-glmer(AB ~ Group + NursStat*Side + (1|Presentation)  + (1|Matriline/MonkeyID), data = dat, family=binomial)
30<-glmer(AB ~ NursStat + Group + Side + (1|Presentation)  + (1|Matriline/MonkeyID), data = dat, family=binomial)
```

Appendix 11:

Assessment of normality of data for the vet stimuli by using a Shapiro-Wilk test.

Aggressive approach, affiliative approach and fear were non-normally distributed whilst self-directed and anxiety and maintenance were normally distributed (Table 9.1).

Table 9.1. Test statistics for normal distribution of behavioural categories. Significance assigned at $p \leq 0.05$*, $p \leq 0.005$**, $p \leq 0.001$***.

Behavioural category	Median (Quartile 1, Quartile 3)	*p*-value
Aggressive approach	0 (0, 0)	$p < 0.001$***
Affiliative approach	0 (0, 0)	$p < 0.001$***
Fear	0 (0, 0)	$p < 0.001$***
Self-directed and anxiety related	350302(275262, 491772)	$p = 0.910$
Maintenance	558726 (237666, 807384)	$p = 0.924$
Aggressive approach pre-habituation	0 (0, 0)	$p < 0.001$***
Affiliative approach pre-habituation	0 (0, 0)	$p < 0.001$***
Fear pre-habituation	0 (0, 0)	$p < 0.001$***
Self-directed and anxiety related pre-habituation	123755 (82043, 241996)	$p = 0.304$
Maintenance pre-habituation	165035 (89765, 349457)	$p = 0.203$
Aggressive approach post-habituation	0 (0, 0)	$p < 0.001$***
Affiliative approach post -habituation	0 (0, 0)	$p < 0.001$***
Fear post -habituation	0 (0, 0)	$p < 0.001$***
Self-directed and anxiety related post -habituation	241842 (163564, 273048)	$p = 0.173$
Maintenance post -habituation	315442 (95178, 500812)	$p = 0.682$

Attention bias scores for the vet stimuli pre- and post-habituation were normally distributed for all habituation groups (Shapiro-Wilk, Table 9.2).

Table 9.2 Significance values for normality distribution of attention bias scores for threat- and non-threat stimuli pre- and post-habituation.

Habituation group	Pre habituation	Post habituation
Control (no habituation)	*p*=0.128	*p*=0.818
Irregular habituation	*p*=0.449	*p*=0.625
Regular habituation	*p*= 0.795	*p*=0.119

The amount of fear behaviours displayed during attention bias testing were normally and non-normally distributed (Shapiro-Wilk, Table 9.3).

Table 9.3. Significance values for normality distribution of fear behaviours displayed during attention bias testing pre- and post-habituation. Significance assigned at $p \leq 0.05$*, $p \leq 0.005$**, $p \leq 0.001$***.

Habituation group	Pre-habituation	Post-habituation
Control (no habituation)	p=0.208	p=0.018*
Irregular habituation	p=0.958	p=0.022*
Regular habituation	p=0.074	p<0.001***

Appendix 12:

Normal distribution of the best fit model of threat – non-threat vet photographs.

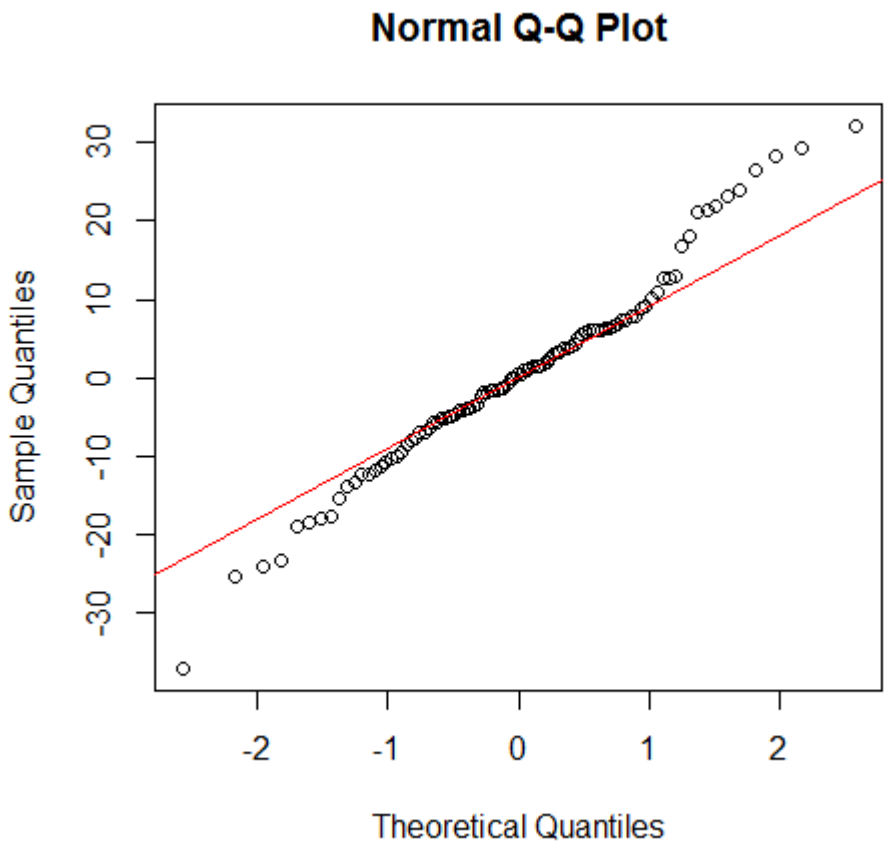


Figure 10.1
Quantile-quantile plots of the best fit model to explain attention bias data of 25 female rhesus macaques, *Macaca mulatta*. Plot shows an interaction between side and age as well as nursing status, *TPH2*, *OPRM1*, *DRD4* and *MAOA*. Lines show line of best fit.