Imprinted genes, Impulsivity and Risk-taking

Claire L. Dent

A thesis submitted to Cardiff University for the degree
of Doctor of Philosophy

School of Medicine
Cardiff University
March 2014
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This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

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Imprinted genes show monoallelic parent-of-origin specific expression and have an important role in mediating adult behaviour. Previous research has indicated that maternally expressed Nesp and paternally expressed Grb10, which are expressed in overlapping brain regions, may have a role in mediating risk-taking and/or impulsive behaviours.

Impulsivity and risk taking are natural parts of human behaviour; however pathological levels of impulsivity and risk-taking are recognised as clinical traits of many psychiatric disorders. The aim of the current research is to explicitly test whether these two oppositely imprinted genes influence impulsivity and/or risk-taking behaviour in mice by examining mouse models that lack functional copies of paternal Grb10 (Grb10+/p) and maternal Nesp (Nesp+/m) in a number of tests of impulsivity and risk-taking. Unlike previous findings in Nesp+/m mice, Grb10+/p mice had the same propensity to explore a novel environment as wild type (WT) controls. However, in a measure of delay-discounting behaviour it was discovered that Grb10+/p mice were less likely to discount delayed rewards. This is in contrast to previous work with Nesp+/m mice, which discounted delayed rewards more steeply. This is the first demonstration that oppositely expressed imprinted genes antagonistically affect behaviour.

To further explore these behaviours, a novel test of risk-taking was developed. Using predator odours a perceived ‘risky’ environment was created in order to measure the decision between fear and reward seeking. Using the Predator Odour Risk-Taking (PORT) task it was demonstrated that Nesp+/m and Grb10+/p mice showed comparable levels of risk-taking behaviour to WT littermates.

Finally, immunofluorescence was used to discover that Nesp55 and Grb10 are not only expressed in the same brain regions, but are co-expressed in some cells, particularly in serotonergic neurons. This suggests that these imprinted genes may be influencing delay discounting behaviour via the same integral neurotransmitter systems.
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<tr>
<th>Abbreviation</th>
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<td>Degrees Celsius</td>
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<tr>
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<td>5 choice serial reaction time task</td>
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<td>Ag</td>
<td>Androgenetic</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>Arc</td>
<td>Arcuate hypothalamic nucleus</td>
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<td>Angelman syndrome</td>
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<tr>
<td>ASR</td>
<td>Acoustic startle response</td>
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<tr>
<td>cDNA</td>
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<td>Choline acetyltransferase</td>
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<td>CMT</td>
<td>Condensed milk test</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>CRF</td>
<td>Continuous reinforcement</td>
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<td>de novo methyl-transferases</td>
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<td>EPM</td>
<td>Elevated plus maze</td>
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<td>Edinger-Westphal nucleus</td>
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<td>Gg</td>
<td>Gynogenetic</td>
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<td>Hypothalamus</td>
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<td>Imprinting centre</td>
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<td>Immunofluorescence</td>
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<td>Immunohistochemistry</td>
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<td>Intraperitoneal</td>
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<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
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<tr>
<td>LacZ</td>
<td><em>Lac</em> operon Z</td>
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<tr>
<td>LC</td>
<td>Locus Coeruleus</td>
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<td>LH</td>
<td>Lateral hypothalamic area</td>
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<td>LMA</td>
<td>Locomotor activity</td>
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<td>Noradrenaline</td>
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<td>Nucleus accumbens</td>
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<td>Sodium chloride</td>
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<tr>
<td>NPP</td>
<td>Novelty place preference</td>
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<tr>
<td>NTC</td>
<td>Non-template control</td>
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<td>OCD</td>
<td>Obsessive compulsive disorder</td>
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<td>OF</td>
<td>Open field</td>
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<tr>
<td>PAG</td>
<td>Periaqueductal Grey</td>
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<td>Polymerase chain reaction</td>
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<td>Positron emission tomography</td>
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<td>Prefrontal cortex</td>
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<td>Parthenogenetic</td>
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<td>PH</td>
<td>Posterior hypothalamic area</td>
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<td>PORT</td>
<td>Predator odour risk-taking task</td>
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<td>PPI</td>
<td>Prepulse inhibition</td>
</tr>
<tr>
<td>PR</td>
<td>Progressive ratio</td>
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<td>Progressive ratio task</td>
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<td>Prader-Willi syndrome</td>
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<tr>
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<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
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<tr>
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<td>Suprachiasmatic nucleus</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNC</td>
<td>Substantia Nigra pars Compacta</td>
</tr>
<tr>
<td>SPA</td>
<td>Subparaventricular zone of the hypothalamus</td>
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<tr>
<td>SPSS</td>
<td>Statistical package for social sciences</td>
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<td>SSRTT</td>
<td>Stop Signal Reaction Time task</td>
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<tr>
<td>SuM</td>
<td>Supra-mammillary nucleus</td>
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<tr>
<td>TBST</td>
<td>Tris-Buffered Saline and Tween</td>
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<td>TE</td>
<td>Tris-EDTA</td>
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<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TS</td>
<td>Turner’s Syndrome</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
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<tr>
<td>VS</td>
<td>Ventral striatum</td>
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<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
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<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>ZI</td>
<td>Zona incerta</td>
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Chapter 1 – General Introduction

1.1 Introduction

Genomic imprinting is an interesting phenomenon in which epigenetic marking of a particular subset of genes results in monoallelic expression, in a parent-of-origin manner. These genes are not only interesting on a molecular level, but also on a systems level, whereby there is increasing evidence to suggest that they have an important role on not just development and growth, but adult behaviour too. This leads to intriguing possibilities about the neural mechanisms of imprinted genes and the behaviours that they regulate. Two examples of imprinted genes are $Nes\rho$ and $Grb10$, which are of particular interest due to their striking overlapping expression in discrete regions of the brain (Plagge et al., 2005, Garfield et al., 2011), which in turn may represent ‘hot-spots’ of imprinted gene expression (Gregg et al., 2010). $Nes\rho$ and $Grb10$ are oppositely imprinted within the brain, whereby $Nes\rho$ is expressed from the maternal allele (Plagge et al., 2005); and $Grb10$ from the paternal allele (Garfield et al., 2011). This expression pattern raises the question as to what role these two genes have in the adult brain, and also raises the hypothesis that these genes may be acting on the same behaviours, possibly antagonistically. This thesis will primarily focus on the functional studies examining whether these two imprinted genes have analogous or antagonistic roles in mediating adult behaviour, specifically in relation to impulsivity and risk-taking.
1.2 Imprinted genes

1.2.1 Introduction to imprinted genes

Imprinted genes represent a unique subset of mammalian genes which, despite having both maternal and paternal alleles present in the genome, are expressed from one parental allele only. Imprinting is a developmentally determined process, established during gametogenesis, whereby one parental allele is epigenetically marked, resulting in monoallelic expression (Wilkinson et al., 2007). Selection of which parental allele is expressed is also determined during development, and results in some imprinted genes being only maternally expressed (e.g. Nesp, Cdkn1c) whilst others are paternally expressed (e.g. Peg3, Igf2). This process occurs in a parent-of-origin manner, whereby the inherited gene maintains a molecular memory (or mark) of its parent-of-origin, resulting in differential transcription of that gene (Figure 1.1). These marks are created through epigenetic processes (commonly DNA-methylation) which regulate the expression of imprinted genes (Holmes and Soloway, 2006). This epigenetic mark is preserved throughout the somatic cells of the progeny; however these marks are erased in the germ cells of the developing embryo and subsequently re-set according to the sex of the developing individual (Delaval, 2004), therefore maintaining the imprint in a parent-of-origin fashion (Figure 1.1). Therefore parental genomes exhibit an epigenetic asymmetry at fertilization, which give rise to functional differences between parental genomes during development, differences which are maintained throughout life (Surani, 2001).
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Figure 1.1 Simple schematic showing the process of genomic imprinting. Parent-of-origin monoallelic expression occurs through marking of either the male or female allele (represented by green lollipops), which occurs through epigenetic mechanisms such as DNA methylation and histone modification. The epigenetic mark established during embryogenesis results in differential transcription, resulting in predominant expression from one parental allele only. The epigenetic marks are then reset during egg and sperm formation. This inherited set of marks is maintained in the somatic cell lineages of the offspring, but in their germ cells the cycle of imprinting repeats, with erasure and re-initiation of the imprint. Therefore in the sperm, whether the gene is inherited maternally or paternally, all imprints are erased and re-written with the paternal pattern of imprinting; likewise in the egg, all imprints are erased and re-written with the maternal imprint. Consequently inheritance of imprinted traits is non-Mendelian, and is instead characterized by passage down one parental line. Image adapted from Wilkinson et al. 2007.

1.2.2 Epigenetic regulation of imprinted genes

Imprinted genes generally occur in clusters (Figure 1.2) within the genome, containing two or more imprinted genes (Bartolomei et al., 2011). Imprinting is an epigenetic process, such that regulation is achieved without altering the nucleotide sequence. The principle epigenetic mechanism for the establishment of imprinting and monoallelic expression at imprinted gene clusters is the parental specific DNA methylation of DMRs (differentially methylated regions) (Constancia et al., 1998). This occurs through the addition of methyl groups to the cytosine nucleotides of CpG islands. (Delaval and Feil, 2004). CpG islands represent areas of the genome that have an elevated G+C base composition compared to the CpG poor landscape of the rest of the genome (Deaton and Bird, 2011). DMRs can be germline (methylation acquired during oogenesis or spermatogenesis) or somatic (methylation acquired after fertilisation) (John and Lefebvre,
Somatic DMRs can also be tissue specific, in turn leading to tissue specific monoallelic expression, as occurs for some imprinted genes. Germline DMRs are essential for the establishment of somatic DMRs, and ultimately for all imprinted gene regulation (John and Lefebvre, 2011). Consequently some are known as ‘imprinting control regions’ (ICRs) or ‘imprinting centres’ (ICs). However, it is now known that the methylation of DMRs in the germline is more widespread and not specific to imprinted loci alone (Smallwood et al., 2011). Germline DMRs are maintained by de novo methyl-transferases (DNMTs), which methylate the CpG sequence (Bartolomei and Ferguson-Smith, 2011). Specifically DNMT1 targets newly synthesized complementary strands containing CpG regions for methylation, which enables germline DMRs to be maintained throughout embryogenesis. This is now thought to be the default marking process, with methylation at DMRs associated with imprinted genes is protected from the genome-wide wave of de-methylation that takes place early during embryogenesis (Kelsey and Feil, 2013). These initial DNA-methylation imprints are subsequently built upon with other modifications in order to preserve the imprinting status. This occurs through a combination of non-coding RNA, additional DNA methylation, changes in histone modifications and higher chromatin structure (Abramowitz and Bartolomei, 2012). This process allows the parental imprints to be relatively stable and robustly maintained.
1.2.3 Functional Importance of imprinted genes

The functional importance of imprinted genes was first discovered during the seminal work of two laboratories, McGrath and Solter (1984) and Surani et al. (1984), who were the first to find evidence for the requirement of both the maternal and paternal genomes for normal development. During this research, Surani et al. (1984) used embryos created from oocytes to which the pronucleus of either another oocyte (therefore diploid for maternal genome only) or a sperm was added. They found that the oocytes which received a male pronucleus developed to term but those with two female pronuclei developed poorly after implantation (Surani et al., 1984); therefore demonstrating that maternal and paternal genomes were not functionally equivalent. It was concluded from this work that during gametogenesis some genes are imprinted with an indication (or mark) of the gamete in which the genes came from. Furthermore observations from work by
McGrath and Solter (1984), indicated that whilst neither gynogenetic nor parthenogenetic (diploid for maternal genome only) nor androgenic (diploid for paternal genome only) embryos were viable, prominent differences in the development of these embryos occurred. Notably that androgenetic embryos showed increased extra-embryonic tissue and foetal growth, whilst parthenogenetic embryos showed decreased extra-embryonic tissue and foetal growth (McGrath and Solter, 1984). This significant finding provided the first evidence that maternal and paternal genomes could differentially regulate gene expression, and subsequently influence cellular function in different ways.

Following the early embryonic work, came the identification of the first paternally expressed (Igf2) and maternally expressed (Igf2r, H19) genes in the mouse (Barlow et al., 1991, Bartolomei et al., 1991, DeChiara et al., 1991). To date there are approximately 150 imprinted genes identified in the mouse (http://www.mousebook.org/catalog.php?catalog=imprinting) with a similar number in humans (Moore and Oakey, 2011). However a recent genome-wide analysis of the embryonic and adult mouse brain revealed more than 1300 potential loci for imprinting (Gregg et al., 2010). However the experimental validity of this study has been subject to criticism, suggesting that only a fraction (approximately 50) of these loci represent novel putative imprinted genes (DeVeale et al., 2012).

Although only a handful of imprinted genes have been fully functionally characterised, with particular reference to their potential effect in the brain, it is clear that imprinted genes influence some key physiologies. The most well studied of these is the role of imprinted genes on in utero growth, placental function and nutrient transfer from mother to offspring (Charalambous et al., 2007, Gutierrez-Marcos et al., 2012). Furthermore it is becoming increasingly apparent that imprinted genes are also important for post-natal functions, such as suckling (Plagge et al., 2004), energy homeostasis (Smith et al., 2006) and, the focus of this thesis, adult behaviour.

1.2.3.1 In utero growth

The importance of imprinted genes on in utero development has been well established (Charalambous et al., 2007, Tunster et al., 2013). Based on the profound effects on extra-embryonic tissue found in the early embryonic work in mice, and the fact that the
majority of the imprinted genes are expressed in the placenta, highlights their importance for *in utero* growth. The two most prominent developmental functions imprinted genes have are nutrient transport and placental signalling (Tunster et al., 2013); both of which are key to the direct and indirect regulation of birth weight. The first imprinted genes to be identified (*Igf2, Igf2r, H19*) were all shown to play a critical role in embryonic development. Specifically *Igf2*, which has four main transcripts (P0, P1, P2, P3) expressed in the foetus and the placenta during development, has been shown to play a central role in the placental nutrient supply to the foetal nutrient demands for growth (Reik et al., 2003). Importantly mouse imprinting knock-out studies have shown that deletions of paternally expressed *Igf2, Ins1/Ins2, Peg1, or Peg3* genes result in intrauterine growth restriction (DeChiara et al., 1991, Duvillie et al., 1997, Lefebvre et al., 1998, Li et al., 1999). Conversely deletion of the maternally expressed *Igf2r or H19* genes, or over-expression of the *Igf2* gene, results in foetal overgrowth (DeChiara et al., 1991). Similarly, a double dose (two copies) of the paternally expressed imprinted gene *Dlk1* results in a growth enhanced foetus even when the placenta expressed normal levels of the gene (da Rocha et al., 2009). Furthermore mice deficient for the maternally expressed imprinted gene *Cdkn1c*, were found to have an initial 20% increase in weight during early gestation but experienced a rapid reversal of this overgrowth phenotype in late gestation, resulting in newborn pups of normal weight (Tunster et al., 2011). This highlighted the role of imprinted genes in the allocation of the maternal resources via the placenta.

The effect of *in utero* growth is not limited to the effects of paternally expressed genes; maternally expressed genes have also been shown to effect placental growth. It was shown that a single extra dose of the imprinted gene *Phlda2* caused a dramatically altered placental structure leading to the restriction of embryonic growth (Tunster et al., 2010). Moreover mice that were null for *Phlda2* and the maternally expressed imprinted gene *p57Kip2* resulted in increased placental size (Takahashi et al., 2000, Frank et al., 2002). Taken together this is indicative that a significant role of imprinted genes is to control nutrient supply via the placenta (Isles and Holland, 2005).

In recent research it has also been found that alterations in the normal expression pattern of the imprinted gene *Igf2*, known for its role in *in utero* development, can also influence anxiety in adulthood (Mikaelsson et al., 2013). Mutant mice null for the placenta
specific transcript (P0) of Igf2, which has been hypothesized to cause an imbalance between foetal demand and placental supply of nutrients, demonstrated increased anxiety in later life, suggesting a role for imprinted genes in the long term programming of emotional behaviour, via altered placental mechanisms (Mikaelsson et al., 2013).

1.2.3.2 Energy homeostasis and adaption to post-natal life

There is increasing evidence to show that imprinted genes do not just affect the nutritional resources of offspring *in utero* but also after birth via direct or indirect postnatal functions. Research has identified that imprinted genes demonstrate a role in prenatal growth, appetite, fat and lean mass deposition, metabolic function, energy homeostasis and insulin sensitivity/production (Charalambous et al., 2007). For example the two reciprocally imprinted genes, *Gnas* (maternally expressed) and *Gnasxl* (paternally expressed) code for the proteins Gsα and XLαs, respectively, and have been found to have an antagonistic role upon glucose metabolism and energy expenditure (Charalambous et al., 2007). Gsα haploinsufficiency causes a reduced sensitivity to insulin which results in obesity and diabetes (Chen et al., 2005). In contrast, XLαs normally acts to reduce signalling to insulin-responsive tissues, and mutant mice null for paternal *Gnasxl* showed impaired regulation of energy homeostasis, and were lean showing hypermetabolism and severely reduced suckling ability (Plagge et al., 2004, Xie et al., 2006).

The imprinted gene *Peg3* known for its role in mediating maternal behaviour, also has an effect on neonatal growth; mutant offspring null for paternally inherited *Peg3* display reduced infant growth, delays in weaning and the onset of puberty (Curley et al., 2004). Furthermore recent research has highlighted the importance of imprinted gene dosage in regulating neonatal body temperature for survival. A mutation in the imprinted gene cluster on mouse chromosome 12, resulting in a triple dose of the imprinted gene Dlk1, caused postnatal lethality, highlighting that control of gene dosage at imprinted loci is essential for the successful transition into independent life (Charalambous et al., 2012). Taken together these findings demonstrate that imprinted genes have a crucial role in the postnatal control of nutritional resources through the central control of energy homeostasis (Bartolomei and Ferguson-Smith, 2011).
1.2.4 Evolution of imprinted genes

Imprinted genes offer an interesting and unresolved evolutionary conundrum, whereby they negate the benefits of diploidy, yet appear to be necessary for normal development (Fowden et al., 2006). Therefore a number of theories have been proposed relating to how and why imprinting may have evolved (Haig, 2000, Wolf and Hager, 2006). Although most research on imprinting has focused on understanding the epigenetic mechanisms of imprinted genes (Ferguson-Smith, 2011), some researchers have attempted to address these ideas by focusing not on the epigenetic mechanisms, but the specific functions of imprinted genes. The effects that imprinted genes have on adult behaviour have prompted the idea that genomic imprinting may be evolutionarily adaptive, with one theory being that this class of genes has arisen in order to coordinate the co-adaptation of viviparity and maternal care in mammals (Keverne and Curley, 2008). One theory known as the ‘placenta hypothesis’ (Kaneko-Ishino et al., 2003) suggests that viviparity is the driving force for genomic imprinting in placental mammals, due to the fact that imprinted genes are found in eutherians (placental mammals) and in marsupials, which have a rudimentary placenta, but are absent in monotreme (egg-laying) mammals and birds (Kaneko-Ishino et al., 2003). The emergence of imprinted genes in placental animals has also been explained by the dynamics of retroviruses in the placenta (Haig, 2012). This highlights that genomic imprinting shares many of the same processes as the suppression of foreign DNA (e.g. retroviruses), such that DNA methylation both inactivates ‘foreign’ DNA and controls imprinted gene expression. It is therefore hypothesized that the ancestral function of DNA methylation is ‘host defence’ and that the reason imprinted genes contain sequences that are subject to methylation is because they look like foreign DNA (Barlow, 1993). This concept therefore argues that DNA methylation is a defence mechanism and therefore that genomic imprinting is an incidental side-effect for genes that happen to look ‘foreign’ (Haig, 2012).

A more widely accepted hypothesis comes from another theory of imprinted gene evolution, namely the intra-genomic conflict or kinship hypothesis (Haig, 2000). The basic concept here is that parental alleles act to promote their own interests and in some circumstances this leads to antagonistic action between maternal and paternal alleles. It is proposed that paternally expressed genes maximise the father’s reproductive success
through promoting embryonic and early post-natal growth by extracting nutritional resources from mother. Maternally expressed genes, in contrast, are thought to resist maternal resources being exhausted on a single pregnancy and ensure some reserves are withheld for subsequent pregnancies and future offspring (Keverne and Curley, 2008). Accordingly, paternally expressed imprinted genes tend to be growth promoting whereas maternally expressed imprinted genes tend to be growth limiting (Haig and Graham, 1991).

One such circumstance where this intra-genomic conflict is illustrated at a physiological level is by the oppositely imprinted genes *Igf2* and *Igf2r*. The insulin-like growth factor type 2 gene (*Igf2*) is paternally expressed and is a critical foetal growth factor, whereas the maternally expressed *Igf2* receptor gene (*Igf2r*) binds to *Igf2* and targets it for degradation, therefore suppressing foetal growth (Haig and Graham, 1991). A similar asymmetry of relatedness arises within a social group when there is sex-biased dispersal (Haig, 2000), and provides a route by which intragenomic conflict could influence behaviour, with a particularly impact upon social behaviour (Isles et al., 2006).

The importance of many imprinted genes, such as *Igf2*, *Peg1* and *Peg3* in placental function and *in utero* growth, has also led to the suggestion that imprinted genes may have arisen as a mechanism to facilitate the co-adaptation of two important mammalian features, viviparity (Wolf and Hager, 2006) and maternal care (Curley et al., 2004). The co-adaptation theory postulates that instead of conflict, maternal–offspring interactions may reflect a co-adaptive integration of the maternal and offspring genomes; therefore developing traits that positively affect offspring development and fitness. Genomic imprinting with maternal expression is therefore most likely to evolve when selection favors co-adapted maternal and offspring traits, for example during mammalian development in utero (Wolf and Hager, 2006). This hypothesis therefore offers a rationale for the high expression of imprinted genes in the placenta. Furthermore the co-adaptation theory offers a potential explanation for the preferential maternal contribution to gene expression as suggested by the recent genome-wide analysis of the murine brain (Gregg et al., 2010).

Whilst the kinship and co-adaptation hypotheses are appealing, both have their limitations and exceptions. For instance, the role of *Peg1* and *Peg3* in maternal care (Úbeda and Gardner, 2011) cannot be explained by the kinship hypothesis. Furthermore the maternal-offspring co-adaptation theory does not easily explain the role of those imprinted
genes not expressed in the placenta and/or having a role in maternal provisioning and/or care, but which show strong expression and are imprinted in the brain. Of those well-characterized imprinted genes (showing an epigenetic mechanism and parent-of-origin specific expression - http://www.mousebook.org/catalog.php?catalog=imprinting) approximately 40% are brain expressed in mice, with prominent examples including \textit{Ube3a} (Rougeul et al., 1997), \textit{LRRMT1} (Francks et al., 2007) and \textit{Inpp5f} \textit{v2} & \textit{Inpp5f} \textit{v3} (Choi et al., 2005).

\textit{1.2.5 Imprinted genes and the brain}

Imprinted genes play a role in a number of key functions and are expressed in various tissues throughout the body. Nevertheless, imprinted gene expression is particularly prominent in the placenta (Gutierrez-Marcos et al., 2012) and the central nervous system (Davies et al., 2005). Imprinted genes have an established role in the placenta in controlling \textit{in utero} development in mammals (Tunster et al., 2013); however a number of imprinted genes are expressed in the brain into adulthood, pointing towards a role in the control of functions beyond early development.

\textit{1.2.5.1 Early Chimera work}

Chimeras provide a useful laboratory method to study the differential effect of the paternal and maternal genome on development. Early work utilising mouse chimeras provided the first clue that imprinting plays a significant role in brain development (Barton et al., 1991, Keverne, 1997). Embryos constructed from a combination of normal cells with parthenogenetic (Pg)/gynogenetic (Gg) and androgenetic (Ag) cells were viable, but required less than 50\% of the embryo to be chimeric to survive (Keverne et al., 1996). Using cells which expressed the marker \textit{LacZ}, allowing the distribution of these cells to be followed, it was found that the contribution of Pg/Gg and Ag cells to brain development was dimorphic (Keverne et al., 1996). Whereby Ag and Gg contribution resulted in differences in brain size, specifically Ag chimaeras had smaller brains, whereas Pg/Gg chimeras had larger brains. As well as overall brain volume, the Pg/Gg and Ag cells were located reciprocally within the adult brain. Although initially distributed throughout the developing brain, Pg/Gg and Ag cells were selectively ‘lost’ from certain regions; leading to consistent populations in distinct
brain regions. Specifically, Ag cells contributed substantially to the hypothalamic structures and not the cortex (Keverne et al., 1996). Conversely, Pg/Gg cells contributed substantially to the cortex, striatum and hippocampus but not to the hypothalamic structures (Keverne et al., 1996). The strikingly contrasting contributions of maternal and paternal cells to telencephalic and diencephalic structures respectively, led the researchers to propose that cells may have functional differences in the brain according to their parent of origin (Keverne, 1997).

1.2.5.2 ‘Maps’ of genomic imprinting

The seminal findings of Keverne et al. (1996) were the first to uncover the patterns of imprinted gene expression in the brain. The discovery that maternal and paternal genomes have dissociable expression in distinct brain systems has been subsequently interpreted to explain the functional differences of the maternal and paternal genomes (Wilkinson et al., 2007). Specifically that the maternal interests are mediated by 'higher' cognitive systems, whereas the paternal interests are mediated by effects on brain systems that operate 'emotional' or 'autonomic' functions (Wilkinson et al., 2007). However without a full understanding of the developmental profiles of all brain-expressed imprinted genes, it is difficult to draw such conclusions and as a result no organisational ‘maps’ of imprinting have emerged. However, a recent genome-wide analysis of parent-of-origin allelic expression illustrated that there are many distinct regions of the brain in which putative imprinted genes are expressed (Gregg et al., 2010). Specifically, 26 out of 118 brain regions were identified as ‘hotspots’ for genes showing a parent-of-origin bias in expression (Gregg et al., 2010). These include the arcuate nucleus, dorsal raphe nucleus, substantia nigra pars compacta, ventral tegmental area, dorsal hypothalamic area, locus ceruleus, and nucleus accumbens. Whereas the expression hotspots of 20 randomly selected control genes with biallelic expression were located predominantly in cortical and olfactory regions and appeared entirely distinct from that of imprinted genes (Gregg et al., 2010). However, it is important to recognise that the genes identified in this study were done so on the basis of a bias in parental allele expression, and do not have a robust associated imprinting mark (i.e. DMR) (Xie et al., 2012). Nevertheless this has made an important contribution to the
existing knowledge of areas of the brain that may be mediated by genomic imprinting. This may shed further light on the functions and behaviours that imprinted genes control.

1.2.6 Imprinted genes and behaviour

The use of mouse models has facilitated the understanding of the role that imprinted genes have on brain and behaviour. Early behavioural experiments using chimeras were the first to discover a role for imprinted genes on behaviour. Behavioural experiments revealed that mouse chimeras high in Pg cells were more aggressive (Allen et al., 1995). Given the importance of olfactory cues in animal aggression, the authors concluded this was as a result of the high levels of Pg cells found in the olfactory bulb. Since this finding there has been further evidence of imprinted genes in mediating olfaction (Isles et al., 2001, Swaney et al., 2007). Moreover, the discovery that Pg cells make a substantial contribution to the hypothalamus; a structure important for autonomic maintenance of homeostasis, means the paternal genome is likely to exert significant control over important behaviours such as feeding, sex, emotional and maternal behaviour (Goos and Silverman, 2001). As well as chimeras, another way of investigating the role of genomic imprinting on behaviour is through targeted gene deletion. An increasing number of behavioural analyses have been performed on various imprinted gene knockout mice. As a result of this, there is evidence that a diverse array of behaviours appear to be influenced by imprinted genes, such as aspects of cognition, emotion and, most extensively, maternal care and mother-infant interactions.

1.2.6.1 Maternal behaviour

The role of imprinted genes in maternal behaviour has been well documented in studies using laboratory animals. The first example of these is the role of the paternally expressed imprinted genes Peg1 and Peg3, which have been shown to have a role in nurturing (Keverne, 2001). Mice null for the paternal allele of Peg1 demonstrated impaired maternal behaviour including reduced pup-retrieval, nest-building and placentophagia (Lefebvre et al., 1998). This effect has also been observed in mice null for the paternal allele of Peg3, where reduced maternal behaviour often resulted in death of the offspring (Li et al., 1999). Similarly, it was found that mutant mothers null for the paternal allele of Peg3
have impaired maternal reproductive success through reduced maternal care, reduced maternal food intake during pregnancy, and reduced milk let-down (Curley et al., 2004). As well as impairments in maternal behaviours, imprinted genes are also critical for mediating maternal effects on offspring development, such as postpartum licking and grooming (Champagne et al., 2009). It is therefore increasingly evident that one area in which imprinted genes play an important role is the interaction between mother and offspring in early postnatal life.

1.2.6.2 Cognition

Exploration of the role of imprinted genes on cognition has been varied, due to the involvement of imprinted genes in syndromes with cognitive deficits. Clinical research carried out in the context of abnormal human conditions such as the X-linked imprinting disorder Turner’s Syndrome (TS) has demonstrated the involvement of imprinted genes in numerous cognitive functions (Isles and Wilkinson, 2000). These functions include behavioural inhibition (specifically the ability to withhold a prepotent response) and behavioural flexibility (Skuse et al., 1997), mental rotation and long-term memory (Bishop et al., 2000); as well as social cognition and visual-perceptual reasoning (Lepage et al., 2012). Strong evidence that imprinted genes affect cognition has also come from studies of Autism spectrum disorder (ASD). ASD, associated with impairments in social interactions and communication, and repetitive and stereotyped behaviour, is highly heritable (Badcock and Crespi, 2006). Genetic linkage studies have identified several genes associated with ASD, a proportion of which are imprinted. Which provides evidence for the notion that genomic imprinting causes a diversity in cognition and manifests as ASD (Badcock and Crespi, 2006).

Preclinical research utilising mice with knock-outs of certain imprinted genes, particularly relating to Prader-Willi Syndrome (PWS), have led to the finding that a spectrum of cognitive functions are influenced by genomic imprinting. These include aspects of learning and memory, for example research using mice deficient for the imprinted gene Necdin showed improvement in particular cognitive functions associated with spatial learning and memory capability (Muscatelli et al., 2000). Furthermore, mice with a deletion of the PWS imprinting centre, showed impaired attentional function in the 5-choice serial reaction time task (5-CSRTT) (Relkovic et al., 2010). Therefore through the study of a
number of disorders, it is becoming increasingly clear that imprinted genes may also contribute to cognitive function.

1.2.6.4 Neurobehavioral disorders

There is a growing association of imprinted genes with neurological disorders and psychiatric illness. Such as the association of the imprinted region 15q11-q13 with the disorders PWS and Angelman Syndrome (AS) (Cassidy et al., 2000). PWS is a neurodevelopmental disorder characterized by an insatiable appetite, dysmorphic features, behavioural difficulties, and mild learning disabilities (Goldstone, 2004). AS is a neurogenetic disorder which results in severe intellectual and developmental disturbances, seizures and ataxia (Cassidy and Schwartz, 1998). Both PWS and AS are disorders of impaired imprinting, that result from the absence or lack of expression of one parental allele of the same region of chromosome 15q. This usually occurs through de novo microdeletion of the 15q11-q13 region from either the paternal or maternal allele (Cassidy et al., 2000). Absence of expression from the paternal allele results in PWS, whilst absence of expression from the maternal allele results in AS; manifesting in two distinct forms of cognitive impairment.

Furthermore altered dosage of imprinted genes is associated with abnormal brain function and neuropsychiatric illness (McNamara and Isles, 2013). Abnormalities in the regulation of the 15q11–q13 imprinting region have been shown to manifest in not just neurodevelopmental disorders such as PWS and AS, but increased incidences of psychosis (Ingason et al., 2011a) and autism (Hogart et al., 2009). Specifically an increased dosage of the maternally derived allele of 15q11–q13 results in increased susceptibility to autism. ASD has also been associated with the dysregulation of the imprinted gene Ube3a (Glessner et al.), as well as pre-clinical research demonstrating that mouse models carrying a ‘double dose’ (two copies) of Ube3Aa results in symptoms typical of psychosis and schizophrenia (Craddock and Owen, 2010). Although less well characterised, parent specific transmission and, by implication, genomic imprinting, has been shown to affect the inheritance of certain disorders such as epilepsy, Tourette’s syndrome and bipolar disorder (Goos and Silverman, 2001). For example women with bipolar disorder are more likely to have children with the condition than are men with the disorder (Keverne, 1997). From the growing repertoire of evidence, it is becoming increasingly clear that imprinted genes are not just important for
the regulation of embryonic development and growth, but are also responsible for multiple human diseases and disorders.

1.2.6.3 Emotional behaviour

The role of imprinted genes on emotional behaviour has been less well studied. However the high presence of paternal imprinted gene expressing cells in the hypothalamus has suggested an involvement of the paternal genome in emotional behavior (Goos and Silverman, 2001). Research into the role of genomic imprinting in emotional behavior has focused on maternal behavior and neurogenetic disorders where abnormal emotion is a symptom, for example AS and PWS (Cassidy and Schwartz 1998). In addition to being associated with neurological and motor impairments, AS is also associated with excessive laughter and smiling (Cassidy and Schwartz, 1998, Cassidy et al., 2000). Furthermore, as well as physical and cognitive symptoms, individuals with PWS also show increased negative affect signals such as stubbornness and temper tantrums (Goldstone, 2004). Although these disorders are distinct, they are both caused by genetic anomalies involving a cluster of imprinted genes on chromosome 15. Therefore through clinical data from neurodevelopmental disorders, it has been shown that imprinted genes have a role in the regulation of emotion. Moreover it has been found that imprinted genes influence emotional attachment between a mother and offspring, via the control of positive affect signaling (Isles and Holland, 2005). More specifically, maternally expressed genes such as UBE3A and ATP10C normally act to limit the amount of positive affect signals (Isles and Holland, 2005).

1.2.7 Nesp

The Nesp transcript is expressed exclusively from the paternal allele, and encodes Neuroendocrine secretory protein 55 (Nesp55), which consists of 244 amino acids (Bartolomucci et al., 2011). Nesp is part of the complex imprinted GNAS gene locus on human chromosome 20q13.2 and mouse chromosome 2 (Figure 1.2), which encodes the α-subunit of the stimulatory G-protein Gsα (Bartolomucci et al., 2011). The biochemical function of Nesp55 is only partially understood but it appears to be related to fast anterograde axonal transport in the peripheral nervous system and represents a novel
peptidergic marker for a large constitutively secreting vesicle pool (Fischer-Colbrie et al., 2002). However, it is unknown whether Nesp55 influences the transport or release of neurotransmitter vesicles (Plagge et al. 2005). The protein can also undergo proteolytic processing resulting in the release of a carboxy-terminal octapeptide, although this process is limited in the brain (Lovisetti-Scamihorn et al., 1999). Previously generated mouse models have revealed an essential role of the Gnas imprinting cluster in postnatal viability, growth, and the regulation of energy homeostasis (Plagge et al., 2004), although Nesp55 itself does not appear to contribute to these functions (Plagge et al. 2005).

The monoallelic expression of Nesp occurs as a result of complex imprinting at the Gnas locus. There is a somatic DMR spanning the promoter of Nesp55, this region is unmethylated in early embryogenesis but by embryonic (E) 10.5 it has acquired paternal promoter-specific differential methylation (John and Lefebvre, 2011). Therefore gametic methylation must occur to spare the Nesp-DMR of the maternal allele from somatic methylation. This suggests that either methylation occurs by default when Nesp55 is not expressed, or alternatively the Nesp antisense transcript may actively induce DNA methylation of the Nesp-DMR, therefore silencing the Nesp55 promoter (John and Lefebvre, 2011).

1.2.7.2 Expression of Nesp55 in the brain

Nesp55 is expressed in endocrine and brain tissues, being neuronal specific (Fischer-Colbrie et al., 2002). The expression of Nesp within the rodent brain is discrete, with a high-level of expression in the hypothalamus, pons and midbrain regions, specifically in the dorsal raphe nucleus (DRN), locus coeruleus (LC) and Edinger-Westphal nucleus (EWN) (Figure 1.3) (Bauer et al., 1999). The presence of Nesp expression in these brain structures, plus its potential role in vesicle formation and/or recycling, suggests a possible involvement in noradrenaline (NA) and serotonin (5-HT) release. No differences in total tissue levels of 5-HT or NA has been detected in Nesp55 deficient mice (Bauer et al., 1999, Plagge et al., 2005), but as yet no direct measurement of neurotransmitter release has been made.
Figure 1.3 Expression of Nesp55 in coronal sections of the adult mouse brain. Research utilising in situ hybridisation found high levels of expression in the locus coerules (LC) the dorsal raphe nucleus (DR), the periaqueductal gray (PAG) and the Edinger-Westphal nucleus (EW). Furthermore high expression levels were found in the hypothalamus, specifically: posterior hypothalamic area (PH); dorsal tuberomammillary nucleus (DTM); dorsomedial hypothalamic nucleus (DM); arcuate hypothalamic nucleus (Arc); lateral hypothalamic area (LH), zona incerta (ZI), suprachiasmatic nucleus (SCh), subparaventricular zone of the hypothalamus (SPa).


1.2.7.3 Behavioural role of Nesp

Although there were no gross alterations in general physiology, previous research utilising adult mice with a deletion of Nesp55 ($Nesp^{m/+}$) revealed an influence of this gene on exploratory behaviour (Plagge et al. 2005). This aspect of behaviour was assessed using two separate behavioural tasks of activity; locomotor activity (LMA) and the open field (Plagge et al. 2005). $Nesp^{m/+}$ mice were shown to have an increased reactivity to a novel environment relative to control mice. Specifically $Nesp^{m/+}$ showed heightened activity on the first day of LMA testing, and increased activity (quadrant crossings) over the duration of the 10-min open field test, which has been interpreted as altered reaction to novelty (Plagge et al. 2005). In an additional explicit test of novelty investigation, the Novelty Place Preference task (NPP), results indicated that although $Nesp^{m/+}$ animals showed increased excitement toward the novel environment (making more entries), when given the choice between a familiar and a novel environment, $Nesp^{m/+}$ mice spent significantly more time in the familiar environment, demonstrating an un-willingness to explore a novel environment (Plagge et al. 2005). This combination of behaviours indicates that Nesp55 has a role in mediating novelty exploration.
The role of Nesp on adult behaviour has been further investigated with the use of a delay discounting paradigm (Isles et al. *unpublished data*); which is the concept of measuring the choice between a small immediate reward and a large progressively delayed reward. This concept has been successfully translated into the ‘delayed-reinforcement task’, which has been utilized with rats (Winstanley et al., 2003) and mice (Isles et al., 2003). The delayed-reinforcement task is used as a measure of impulsivity, specifically ‘impulsive choice’, whereby selection of the small immediate reward is indicative of impulsive, and preference for the large reward is indicative of self-control (Winstanley et al., 2004). In previous research it was found that Nesp<sup>m/+</sup> mice exhibited significantly altered delay-discounting behavior in the delayed reinforcement task (Isles et al., unpublished research), specifically showing an increased reluctance to wait for a larger reward, and instead preferring immediate gratification (Figure 1.4). The behaviour being measured in the delay-discounting task is widely accepted as impulsivity (Winstanley et al., 2003), however some argue that delay-discounting behaviour can also be interpreted as risk-taking behaviour (Kalenscher, 2007). This interpretation is due to the theory that in the wild animals would perceive a delay in receiving food as a risk (due to risk of predation or loss of food) therefore the immediate reward is the ‘safer’ option (Kalenscher, 2007). Based on this interpretation, and taken together with behaviour in the LMA, NPP and open field tests, it has been suggested that Nesp55 normally acts to promote ‘risk-taking’ behaviour (Plagge et al., 2005, Isles et al., 2006).
Figure 1.4 Performance of Nesp<sup>m/p+</sup> animals on a delay-discounting test of choice impulsivity. In previous research (Isles et al. unpublished data) Nesp<sup>m/p+</sup> mice were tested in the delayed-reinforcement task; under increasing delay conditions (a) all subjects switched their responding to the smaller rewards associated with a fixed delay (1s). However, Nesp<sup>m/p+</sup> animals were quicker to discount the large reward against the delay than controls. This demonstrates that Nesp<sup>m/p+</sup> mice are significantly more impulsive (or less risk-taking) than WT animals, which suggests that the Nesp55 protein serves to moderate impulsive and/or risk-taking behaviour. Importantly when the large reward was associated with a fixed delay and remained at 1 second throughout the session, Nesp<sup>m/p+</sup> mice maintained a preference for the large reward (b). This demonstrates that the effect found was not a result of satiety or fatigue. Data shown are the mean ± SEM. Control N=7, Nesp<sup>m/p+</sup> N=9.

1.2.8 Grb10

Grb10 encodes growth factor receptor-bound protein 10, which is a cellular adapter protein lacking intrinsic enzymatic activity (Holt and Siddle, 2005). Grb10 is known to interact with numerous tyrosine kinases and signalling molecules, as well as several intracellular proteins (Blagitko et al., 2000), and has been suggested to function in diverse cellular processes; including the regulation of cellular growth and metabolism, apoptosis and cell migration (Holt and Siddle, 2005). Most notably Grb10 has been implicated in binding to, and regulating signals from, the IR (insulin receptor) and IGF1R (type 1 insulin-like growth factor receptor) (Holt et al., 2009). Grb10 shares structural homology with Grb7 and Grb14 but is the only family member of the Grb genes subject to imprinted regulation (Holt and Siddle, 2005). Grb10 is also unique in its allele-specific expression in different tissues, whereby the maternal allele dominates expression but is confined to peripheral tissues outside of the CNS; whereas the paternal allele is exclusively expressed within the CNS, particularly within the brain (Garfield et al., 2011). As well as having allele-specific
expression, the two parental alleles of *Grb10* seem to have distinct functions. Maternal *Grb10* has a role in the regulation of fetal growth (Charalambous et al., 2010), and also influences insulin signalling and fat deposition during adulthood (Holt et al., 2009). Paternal *Grb10* however, has no obvious effects on growth but has been shown to influence facets of behavior.

The allele-specific expression of *Grb10* is regulated by epigenetic mechanisms that occur during embryogenesis that differentially mark the parental alleles in order to make them active or repressed (Sanz et al., 2008). Epigenetic marking occurs at Imprinting Control Regions (ICRs), which regulate imprinted genes through DNA methylation and differential histone modifications (Sanz et al., 2008). During the onset of neurogenesis (between E11.5 and E14.5) the ICRs are marked by differential histone modifications, resulting in a brain-specific loss of a repressive histone modification (H3K27me3) from the paternal *Grb10* allele (Sanz et al., 2008). This loss of H3K27me3 from the promoter region of the *Grb10* paternal allele-specific transcripts leaves a permissive histone mark on the paternal allele (H3K4me2). In the maternal allele however, this region contains two repressive histone modifications (H3K9me3 and H4K20me3)(Sanz et al., 2008). This epigenetic regulation established early on in embryogenesis, results in the allele-specific expression of *Grb10* in different tissues, and is maintained throughout development.

1.2.8.2 Expression of paternal *Grb10* in the brain

Paternal *Grb10* shows a discrete pattern of expression within the brain and, with a strikingly similar distribution to *Nesp*, is located in the hypothalamus, EWn, DRN and LC (Garfield et al., 2011). Additionally, paternal *Grb10* expression is seen in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNC) (Figure 1.5) (Garfield et al., 2011). In the brain *Grb10* also appears to be limited to neurons, as cellular expression of *Grb10* is co-localised with serotonin (5-HT), the dopamine transporter (DAT) and choline acetyltransferase (ChAT)(Garfield et al., 2011). Like *Nesp*, this localisation suggests that *Grb10* may exert an effect on behaviour via important neurotransmitter systems.
1.2.8.3 Behavioural role of Grb10

Behavioural studies of mice with a paternally inherited null Grb10 (Grb10\(^{+/p}\)) demonstrated a role for this gene in social dominance (Garfield et al., 2011). The ‘tube test’, a measure of social dominance, forces an encounter between two unfamiliar animals. During the test animals are released simultaneously at opposite ends of a clear, narrow tube which allows one mouse to pass through, but is not big enough for two mice to pass. The more dominant mouse will exhibit signs of aggression which will force the ‘opponent’ out of the tube (Garfield et al., 2011). In this task Grb10\(^{+/p}\) mutant mice were found to be significantly less likely to back down than their WT opponents. The same observation did not occur with Grb10\(^{m/+}\) mutant mice, suggesting that specific deletion of the paternal allele caused an increase in social dominance. Converging evidence for a role of Grb10 in social dominance was found from patterns of whisker barbering (Garfield et al., 2011). Cages containing at least one Grb10\(^{+/p}\) mutant were significantly more likely to have cage mates whose faces showed evidence of barbering. Importantly when the Grb10\(^{+/p}\) mouse was socially isolated, the remaining cage-mates re-grew their whiskers, suggesting that Grb10\(^{+/p}\) mice were carrying out the barbering of their cage mates (Garfield et al., 2011). Social barbering is considered a robust correlate of social dominance (Long, 1972, Koh et al., 2008), which is consistent with the results obtained from the tube test. Social dominance is
an important aspect of animal behaviour, whereby the establishment of dominance hierarchies is thought to confer major advantages for health, survival and reproductive success (Wang et al., 2011). Competing for social dominance to gain access to limited resources and mating opportunities, is not only an important part of animal behaviour, but may also be correlated with the propensity to take risks (Davis et al., 2009).

1.2.9 Summary of Imprinted gene research

The functional role of imprinted genes remains a fascinating phenomenon. Although understanding of genomic imprinting has developed considerably, the reason for the existence of imprinted genes remains conjecture until their function in the brain and body has been fully characterised. Recent advances have suggested that there may be significantly more imprinted genes in the genome than first thought; this coupled with their growing association with psychiatric illness and important role in development leave intriguing possibilities about what exactly imprinted genes do, and why they exist. The advances in targeted mutagenesis have provided an excellent opportunity to investigate these genes further, and specifically how they might control behaviour. Research employing Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice, have begun to uncover a fascinating role for these imprinted genes in mediating adult behaviour. Whereby Nesp\textsuperscript{m/+} mice show altered behaviour in tests of novelty exploration (Plagge et al., 2005) and delay-discounting (Isles et al. unpublished data), and Grb10\textsuperscript{+/p} mice show increased social dominance (Garfield et al., 2011). This existing research has alluded to both of these genes having an involvement in impulsivity and risk-taking based behaviour. Furthermore the kinship theory of imprinted genes, which proposes antagonistic roles of maternal and paternal alleles, would suggest that Nesp and Grb10 may be controlling this behaviour in opposing ways. The preliminary findings are consistent with this hypothesis, however further investigation is warranted, using more explicit tests of impulsivity and risk-taking.
1.3 Impulsivity and risk-taking behaviour

The term ‘impulsivity’, defined as ‘action without adequate forethought’ (Winstanley et al., 2006), is a natural part of human behaviour. It is a multifaceted behavioural construct which encompasses a variety of underlying behaviours, such as ‘actions that are poorly conceived, prematurely expressed, unduly risky, or inappropriate to the situation and that often result in undesirable outcomes’ (Evenden, 1999). As a result of clinical and pre-clinical research it appears the heterogeneous underlying traits of ‘impulsivity’ are dissociable both behaviourally and neurologically; whereby separate aspects of impulsivity are underpinned by distinct biological mechanisms. These types of impulsive behavior can be broadly divided into two categories: ‘impulsive choice’ (impulsive decision-making) and ‘impulsive action’ (motor impulsivity) (Winstanley et al., 2004). Impulsive choice refers to actions that fail to take into account other possible options or outcomes, and hence may be sub-optimal; impulsive action refers to actions that are premature or difficult to suppress (Dalley and Roiser, 2012).

Another aspect of impulsive choice is risk-taking, which is a complex construct that often overlaps with impulsivity (Xu et al., 2013), as well as the broader subject of decision-making (Rivalan et al., 2013). Although risk-taking refers to a slightly different aspect of behaviour to impulsivity, weighing up possible outcomes to make a choice is fundamental to both impulsive choice and risk-taking. Furthermore impulsive choice and risk-taking are both prevalent in certain psychiatric conditions and their pathology often go hand-in-hand. Risk-taking refers to the trade-off between a cost and a benefit, and the ability to assess risk appropriately is a requirement for survival in both humans and animals (Simon et al., 2009). This involves the assessment of the value of a potential gain, against the risk of a potential loss; such as the probability of death or harm. Risk-taking can occur in a variety of domains, including financial, health/safety, recreational and social, therefore ‘risky’ activities in humans could include such things as hazardous driving, parachuting, bungee jumping, and unprotected sex, as well as gambling and the use of psychoactive substances (Laviola et al., 2003). Both humans and animals have individual differences in the degree to which they are willing to participate in sensation-seeking or risk-taking behaviours (Isgor et al., 2004). Risk-taking therefore encompasses a spectrum of behaviour, whereby individuals can be
characterised from ‘risk-avoiding’ (avoiding behaviours due to the potential risk) to ‘risk-seeking’ (taking part in behaviour due to the risk involved) (Schonberg et al., 2011).

Impulsivity and risk-taking are normal aspects of life, and are therefore adaptive functions necessary for survival (Humphreys et al., 2013). However it seems that in certain individuals these behaviours can become pathological and result in negative consequences. Deficits in impulsivity and risk-taking have been recognised as a clinical trait of many psychiatric disorders, including schizophrenia, attention deficit/hyperactivity disorder (ADHD), obsessive compulsive disorder (OCD), mania and antisocial behaviour. As well as these disorders, the Diagnostic and Statistical Manual of Mental Disorders (DSM) IV also recognises a specific group of impulsive psychiatric illnesses known as ‘impulse control disorders’, which includes intermittent explosive disorder, pyromania, kleptomania and trichotillomania (American Psychiatric Association, 2013). Impulsivity and risk-taking behaviour also have important implications for drug-seeking behaviour and addiction whereby deficient ‘inhibitory control’, a feature of impulsivity, is recognised as an integral element of drug addiction in rodent models (Belin et al., 2008) and human studies (Mitchell, 2004).

The sub-categories of impulsivity are operational in that we have distinct tasks that measure impulsive choice and impulsive action, as well as tasks to measure risk-taking. Measuring impulsivity and risk-taking behaviour in humans has been successfully established using a number of tasks. With the increased recognition of impulsivity and risk-taking as traits of psychiatric illness, these tasks have proved extremely useful in identifying and understanding disorders such as ADHD in children. The pursuit to identify the neurological basis and possible genetic predispositions of psychiatric illness has led to the increasing use of genetically modified animals in measuring behaviour. Therefore many neuropsychological tasks used to measure impulsivity and risk-taking in humans, have been successfully translated for use with laboratory animals.

1.3.1 Choice impulsivity and risk-taking

‘Choice impulsivity’ refers to the decisional aspect of impulsivity, as opposed to the motoric aspect described by impulsive action. Although all impulsivity involves an impulsive
action of some sort, the crucial difference conceptually is that, unlike for measurements of impulsive action, there is no “pre-potent” response that needs to be inhibited. Instead, impulsive choice reflects the decision-making processes rather than motoric inhibition (Winstanley et al., 2006). Choice impulsivity involves an abnormal level of delay aversion, as exemplified by increased preference for immediate reward over more beneficial but delayed reward (Brevers et al., 2012). This is operationalised by impulsive decisions resulting from a distorted evaluation of delayed or probabilistic consequences of behaviour and an increased preference for (smaller) immediate or frequent rewards over more beneficial (larger) delayed or infrequent rewards (Broos et al., 2012). Similarly, risk-taking, which also involves an action, refers to the weighing up of options and consequences in order to make a decision. The ability to appropriately assess cost vs. benefit analysis is essential in both impulsive choice and risk-taking. The inability to accurately assess cost vs. benefit results in maladaptive risk-taking behaviour, which can be dangerous and harmful (Steinberg, 2008).

Much like impulsive action, impulsive choice and pathological risk-taking are significant symptoms in many psychiatric illnesses, it is therefore important to understand the neurological underpinnings of the dissociable aspects of impulsivity and risk-taking, and how alterations in the neurochemical systems involved can be treated.

1.3.1.1 Assessing discounting behaviour

One of the most prominent features of impulsive choice is an intolerance of the delay of a reward or gratification. The method used to target the measurement of this behaviour is the ‘delayed-reinforcement task’, part of the ‘delay-discounting’ paradigm used to measure impulsivity. This has been utilised extensively in humans (Crean et al., 2002), as well as successful translation of paradigms to laboratory animals such as rats (Evenden and Ryan, 1996), and mice (Isles et al., 2003). In animals, delay discounting tasks measure impulsivity using a system in which subjects have to choose between rewards that are relatively small but available immediately, and rewards that are larger but progressively delayed. In the context of delay discounting, impulsive choice is generally considered as an increased preference for immediate over delayed outcomes, even where the delayed outcomes are more advantageous (Mar and Robbins, 2001). Therefore the selection of smaller immediate reward in preference to larger delayed reward has been considered to
reflect ‘impulsive’ choice, whereas the opposite bias toward delayed gratification has been taken to indicate increasing ‘self-control’. The delay-discounting task has been used extensively in behavioural research to measure impulsive choice, and has been useful in drug and lesion studies to further understand neurological underpinnings and therapeutic advances (Cardinal et al., 2000, Cardinal et al., 2001). For example the administration of amphetamine and atomoxetine (which upregulate dopamine and noradrenaline, respectively) have both been shown to increase the choice of the large reward in the delay discounting task (therefore decreasing impulsivity) (Winstanley et al., 2003, Robinson et al., 2008). Furthermore recent research has shown that impulsive choice, as measured by delay-discounting, and impulsive action, as measured by 5-CSRTT or the stop signal reaction time task (SSRTT), do not correlate in rodents or humans, strengthening the assertion that these are dissociable aspects of impulsivity (Broos et al., 2012).

1.3.1.2 Assessing risk-taking

Risk-taking or ‘risky decision-making’ can be defined and interpreted in numerous ways. For example neuroeconomists define risk in terms of the variability of possible outcomes, whereas clinicians and lay people tend to view risk as exposure to possible loss or harm (Schonberg et al., 2011). This spectrum of definition has led to a broad range of paradigms being developed to measure risk taking in humans. Furthermore many of the behavioural tasks used to measure risk-taking in humans have successfully been translated for use with laboratory animals, particularly rodents. As well as rodent analogues of human tasks, there is an increasingly recognised importance for the development of rodent-specific risk-taking tasks, in order to understand the neurological and genetic underpinnings of risk-taking.

The development of animal models of risk taking is critical for understanding the pharmacological and neurobiological substrates underlying the processing of risk and reward. Additionally, such models could be important for the development of treatment to combat the maladaptive risk-taking that frequently occurs in psychiatric conditions. The simplest ways of measuring risk-taking in animals are tasks which measure spontaneous activity and reaction to novel environments (Piazza et al., 1990, Palanza et al., 2001). For example tasks such as the locomotor activity task (LMA), or tests of anxiety such as open
field test (OF), elevated plus-maze (EPM) and novelty place preference (NPP) can measure the degree to which animals will seek novelty, through the way they explore an unfamiliar environment. Rodents have an innate fear of open spaces, due to their association with the risk of danger (Reul, 2014). Therefore, rodents stereotypically demonstrate two conflicting tendencies: desire to explore novel environments, and avoidance of open spaces (Dulawa et al., 1999). Deviation from this typical pattern of behaviour can be indicative of altered levels of anxiety, fear and risk-taking behaviour. For example, this has been demonstrated with mice lacking a functional copy of the imprinted gene Nesp, which demonstrated heightened locomotor activity in the LMA task, increased activity in the OF, and altered exploration of the novel environment in the NPP. Taken together these behavioural findings can be interpreted to suggest that Nesp normally acts to moderate ‘risk-taking’ behaviour (Plagge et al., 2005).

As well as basic tasks that measure spontaneous risk-taking behaviour, many of the paradigms used to measure risk-based decision making in humans have been successfully translated into tasks for use with laboratory animals. Including rodent analogues of the balloon analogue risk task (BART) (Ashenhurst et al., 2012) and the Iowa gambling task (Zeeb et al., 2009). Probabilistic-discounting tasks, used to measure risk-taking behaviour in humans, refer to the observation that a probabilistic gain is considered to be worth less than the same amount of gain available for certain (e.g. £25 vs. 25% chance of £100) (Shead and Hodgins, 2009). This paradigm has also been translated for use with rodents in order to measure risk-taking behaviour (St Onge and Floresco, 2008). Although delay-discounting tasks have been successfully and widely used to measure impulsivity, increasing research suggests that in animal behaviour temporal discounting is a facet of risk-taking (Hayden and Platt, 2007, Kalenscher, 2007). Therefore, when using this task with animals, choice of the temporally distal reward would be considered risky, and the temporally proximal reward is more favourable (Hayden and Platt, 2007, Kalenscher, 2007). This is explained by the postulation that animals associate the delay with collection as risky, this is because in the context of foraging behaviour, delaying the collection of a reward could result in loss of that reward (another animal taking it) or harm (predation) (Kalenscher, 2007). Therefore a temporally distal reward is perceived as being the same as an uncertain reward, which is less desirable to animals due to their risk-averse nature. Consequently in delay discounting
tasks, the preference for the small immediate reward which would be usually categorised as impulsive choice, is regarded as less risk-taking. Therefore impulsivity and risk-taking are opposite behaviours in this task, whereas in the investigation of novelty for instance, an animal that appears impulsive (going into the novel environment more) would also appear to take more risks.

As well as animal analogues of human tasks, recent experimental paradigms have led to the development of novel techniques that allow the characterisation and measurement of risk-taking behaviour specifically in laboratory animals. For example rodent-specific tests, such as the risky-decision making task (Simon and Setlow, 2012) which presents rats with the choice between pressing a lever to obtain a small, “safe” food reward and a large food reward associated with risk of punishment (footshock). As well as tasks taxing regulatory focus (Franks et al., 2012) which evaluate risk-taking in rats by measuring how much they engage in promotion focus (pursuing gain) or prevention focus (pursuing safety). However whether or not these existing tasks truly model a ‘real-life’ risk-taking behaviour that is ethobiologically relevant to rodents is uncertain, and is discussed in this thesis. Nevertheless, development of animal models of risk-taking behaviour that are both reliable and possess face validity is critical for understanding the pharmacological and neurobiological substrates underlying the integration of risk and reward (Simon et al., 2011).

1.3.1.3 Neurobiology of impulsive choice and risk-taking

Due to the multi-faceted nature of impulsivity, deciphering the exact neurochemical processes that underpin the separate facets of impulsivity and risk-taking is a challenging process and therefore remains relatively unclear. Nonetheless it is thought that the dissociable aspects of impulsivity have independent but potentially overlapping neurobiological substrates (Evenden, 1999), and the role of the monoaminergic corticostriatal systems have been largely implicated (Jupp et al., 2013). Analysis of the neurochemical underpinnings of impulsive choice have revealed a role for the same neurotransmitter systems that are thought to mediate impulsive action, such as serotonin (5-HT), dopamine (DA) and noradrenaline (NA) (Winstanley et al., 2006). Investigation into the neurological pathways that control impulsive choice have principally used temporal or reward discounting paradigms. The roles of DA and 5-HT in controlling impulsive choice
have been demonstrated using pharmacological methods, in clinical and preclinical research. DA is heavily implicated in impulse control disorders, and the therapeutic effects of amphetamine in ADHD have been largely attributed to its ability to potentiate dopaminergic signalling (Zeeb et al., 2010). Both amphetamine and methylphenidate have previously been shown to decrease impulsive decision-making on delay-discounting tasks in rats (Winstanley et al., 2005, van Gaalen et al., 2006). Furthermore, the systematic administration of DA antagonists has been shown to increase delay discounting behaviour in rats (Zeeb et al., 2010). Lesions to the 5-HT system clearly effect impulsive action (Harrison et al., 1999, Winstanley et al., 2004), however the effect of global 5-HT depletion on impulsive choice is less clear (Winstanley et al., 2004). Although it has been shown that decreased 5-HT, via subcutaneous injections of SER-082 (5-HT2C,B antagonist) significantly increased preference for the large reward in a delay-discounting task (Talpos et al., 2006). Furthermore administration of a 5-HT1A receptor agonist has been found to increase impulsive choice on the delay-discounting task (Winstanley et al., 2005). Pharmacological evidence has therefore revealed a role for DA and 5-HT in mediating impulsive choice, and furthermore that these neurotransmitters have a receptor-specific effect. Therefore drugs which act as selective agonists or antagonists for particular receptor subtypes can therefore have very different effects on impulsivity (Winstanley, 2011).

The exact neuroanatomical structures of the brain that control impulsive choice remain uncertain. However, the nucleus accumbens (NAcb) along with its cortical afferents, such as the medial prefrontal cortex (mPFC) and anterior cingulate cortex (ACC), are known to play an important part in the motivational aspect of impulsivity and decision-making (Cardinal et al., 2001). Experiments in animals have implicated a network of limbic-striatal brain structures in the regulation of decision making and impulsive choice. Evidence is perhaps strongest for the mPFC, as well as the ventral striatum (VS) and dopaminergic innervation from the ventral tegmental area (VTA) (Setlow et al., 2009). Lesions within both of these systems produce profound alterations (either increases or decreases) in impulsive choice (Mobini et al., 2002, Winstanley et al., 2004b). Fronto-striatal systems have been heavily implicated in the control of numerous aspects of impulsivity (Dalley et al., 2008). Specifically it has been suggested that projections to the Locus Coeruleus (LC) via the NA system from the ACC and orbitofrontal cortex (OFC), has control in the ‘effortful processing’
involved in decision-making processes (Dalley et al., 2008). Research assessing the effect of lesions to specific brain regions has revealed that impulsive choice, like other aspects of impulsivity/decision making, is regionally dependant (Jupp et al., 2013). For example the role of the NAcbs in mediating impulsive choice, whereby excitotoxic lesions of the NAcbs core sub-region results in a significantly increased desire for the small immediate reward (Cardinal et al., 2001a), whilst conversely, lesioning the NAcbs shell does not have an effect on this behaviour (Pothuizen et al., 2005). However, this is unsurprising given that the NAcbs core is part of a larger neuronal network including the amygdala and PFC, which have an established role in the control of impulsive behaviour (Winstanley et al., 2004b). Lesion studies have also provided evidence for the role of the OFCs and basolateral nucleus of the amygdala (BLA) in mediating impulsive choice (Winstanley et al., 2004b). Rats with BLA lesions showed increased choice of the small immediate reward (increased impulsivity) in a delay discounting task, whilst, OFC lesions increased preference for the large delayed reward (decreased impulsivity) (Winstanley et al., 2004b). More specifically, studies using lesions to sub-regions of the OFC have revealed that medial OFC-lesioned rats showed increased preference for the large reward in a delay-discounting task, conversely lateral OFC-lesioned rats showed decreased, preference for the larger-delayed reward (Mar et al., 2011). Furthermore the DRN has also been implicated in the regulation of impulsive choice behaviour and specifically in waiting for rewards (Miyazaki et al., 2012). Specifically suppression of 5-HT neural activity in the DRN impaired patience for delayed rewards in a delay discounting task (Miyazaki et al., 2012). Therefore, despite gross similarities in the regions which regulate decision-making behaviour, research suggests there are specific sub-regions that control impulsive choice behaviour, most notably the DRN, OFC, BLA and NAcbs core (Winstanley et al., 2004b).

The underlying neurobiology of risk-taking and how dysfunction may arise is not completely understood (Llewellyn, 2008), however the systems involved are analogous with those that control impulsive choice. Neuroimaging studies have proven extremely useful in highlighting areas key to modulating risk-taking behaviour in humans. Generally, studies have demonstrated frontostriatal activation during risky decision-making and that subcortical-cortical networks including multiple prefrontal, parietal, and other limbic regions are involved. The processing of risk based decision making can be divided into gains
(reward) and losses (cost), with suggestions that perhaps different neurological regions govern these two separate constructs (Floresco et al., 2008). Reward is a particularly salient feature of any decision-making process, and has been the focus of a lot of research investigating the neural substrates of risk-taking. Whereby the PFC has been heavily implicated in regulating reward-seeking behaviour; lesions to the PFC in rodents has been shown to induce increased risk-taking behaviour (Floresco et al., 2008). For example the striatum, the input structure of the basal ganglia, is thought to be the circuit responsible for mediating goal-directed behaviour, and therefore the ‘reward’ aspect (Delgado, 2007). Specifically it is thought that DA rich mesolimbic regions including the midbrain, striatum, and frontal cortex, have been suggested to play a particular role in processing the reward aspect during decision-making under risk (Rao et al., 2008). However other studies using fMRI have not found separate brain systems for governing gains and losses, but found areas in the brain, including the ventral striatum (VS), ventromedial prefrontal cortex (VMPFC), ventral anterior cingulate cortex (ACC), and medial orbitofrontal cortex (OFC), that were sensitive to the both gains and losses (Rao et al., 2008). Activation in these areas increased with increasing potential gains and decreased with increasing potential losses (Rao et al., 2008).

Neurochemically, the importance of the DA system in the modulation of risk-taking has been widely acknowledged, and has been the subject of a great deal of research into the neurobiology of risk-taking behaviour. These studies suggest that increased DA activity may impair risk-based decision making (St Onge and Floresco, 2008). Animal studies making use of lesioning, and pharmacological exploration have made significant advances in our understanding of how DA modulates risk and reward. Specifically, research has evidenced that neural activity is adaptive, and therefore activity in dopaminergic neurons increases with the magnitude of anticipated rewards (Tobler et al., 2005), and that reward induces dopamine release and activation in the NAc. Furthermore, blockade of DA receptors reduces the preference for rodents to either wait longer or work harder to obtain a larger reward (Cardinal et al., 2000, Salamone et al., 2001). However which specific DA receptors are responsible for risk-taking behaviour remains unclear. Some have argued that agonists more selective for D₃ receptors, are more likely to induce gambling behaviour (Szarfman et al., 2006), whereas others have reported that D₁/D₂ receptor agonists can also stimulate
these effects (Lu et al., 2006); whilst a recent study using PET scans in humans reports that striatal D$_2$/D$_3$ receptors are the most important in the modulation of risk-taking behaviour (Kohno et al., 2013). This is also supported by a study of risk-taking in rats, using pharmacologically induced dopamine receptor activation, it was shown that risky decision-making was attenuated by D$_2$, but not D$_1$ receptor activation (Simon et al., 2011b). Existing research therefore suggests that dopamine signalling in prefrontal cortical-striatal circuitry is key to the integration of reward information with risk information; but moreover that the exact neural substrates of decision-making may vary in the striatal dopamine system as a function of individual differences, and also as a function of the type of risk-taking being measured (Kohno et al., 2013).

1.3.2 Impulsive action

*Impulsive action* is the motoric (as opposed to decisional) aspect of impulsivity, and refers to action without adequate forethought (Winstanley et al., 2004a). Impulsive action is thought to be underpinned by poor ‘impulse control’, which is described as “an active inhibitory mechanism which modulates the internally or externally driven pre-potent desire for primary reinforcers such as food, sex or other highly desirable rewards” (Winstanley et al., 2006). Behaviourally this is operationalised as the inability to withhold a pre-potent response, thereby reflecting poor response inhibition. Impulsive action is both behaviourally and neurobiologically distinct from impulsive choice, where dysregulation of impulsive action results in clinical disorders where diminished inhibitory control is a feature (Brevers et al., 2012).

1.3.2.1 Measuring impulsive action in rodents

Although no tests of impulsive action are used in this thesis, it is important that a brief overview is provided to demonstrate the contrast with tasks of impulsive choice, below. Numerous tasks have been developed to measure impulsive action. The majority of these tasks take advantage of the enhanced stimulus-control of operant methods, but some maze-based approaches have also been deployed successfully to examine this dissociable aspect of impulsive behaviour. ‘Response inhibition’ is the main feature of impulsive action, and refers to the ability to exert executive control over automatic response systems during
the decision-making process. ‘Go/No go’ tasks specifically target the measurement of this aspect of motor impulsivity (Eagle et al., 2008). This is achieved by measuring the ability to inhibit a pre-learnt response, for example making a motor-response to a certain stimuli (‘go’ trial) and refraining from making the motor response to a stimulus during a ‘no-go’ trial (Eagle et al., 2008). This basic concept has been further developed through the ‘Stop signal reaction time task’ (SSRTT), which measure the ability to stop or cancel a motor action once started in response to a ‘stop-signal’ (Humby et al., 2013). This has been used widely in the clinical setting to measure impulsivity, for example, children with ADHD are slower to inhibit their responses than normal children, as indicated by increases in their stop signal reaction time e.g. Purvis and Tannock (2000) and similarly fail to inhibit their “go” response on the “no-go” trials in go/no-go tasks. The SSRTT has been successfully translated for use with rat models (Eagle and Robbins, 2003) and more recently mouse models (Humby et al., 2013).

The 5-Choice Serial Reaction Time Task (5-CSRTT), originally developed to assess attentional and executive functions, can also be used to measure motor impulsivity. This paradigm requires subjects to detect and respond to brief flashes of light presented in a pseudorandom order in one of five spatial locations, in order to receive a reward (Robbins, 2002). Increased instances of premature responding in this task are indicative of impulsive action.

1.3.2.2 Neurobiology of impulsive action

Many of the same systems that control impulsive choice have been implicated in controlling impulsive action, although there are some dissociable aspects (Evenden, 1999). Research has identified the importance of the monoamine neurotransmitters 5-HT and DA in regulating impulsivity (Dalley and Roiser, 2012). Dysregulation of these monoamines are associated with various psychiatric disorders, and are known to manifest in impulsive behaviours, such as aggression, suicide and gambling (Dalley and Roiser, 2012). Specifically, DA is thought to regulate the cognitive aspects of impulsive action, such as attention and reward processing (Bevilacqua and Goldman, 2013). Pharmacological studies, have also found that enhancing DA signalling increases impulsive responding, whilst administration of D2-like receptor agonists reduces impulsivity in rats exhibiting impulsive behaviour on the 5-CSRTT (Fernando et al., 2012).
The importance of 5-HT was first identified through psychopharmacological manipulation of rats in assays of anxiety (Soubrie, 1986). This demonstrated that administration of drugs which decreased 5-HT transmission resulted in decreased behavioural inhibition. Since this discovery extensive research has been conducted in an attempt to understand this process, and it has been found that the neurological regions known to regulate impulsivity are dense in 5-HT and DA releasing neurons (Harrison et al., 1997). Animal studies have provided support for the role of 5-HT and DA in modulating impulsive action. Depletion of DA in the ventral striatum of rats, including the NAc, reduced impulsivity on a 5-CSRTT (Cole and Robbins, 1989). Furthermore drugs that increase brain DA neurotransmission (e.g., methylphenidate or amphetamine) produce profound effects on impulsive behavior, whereby they act to improve stopping performance on the SSRTT and reducing delay-discounting impulsivity, whereas they increase impulsivity in the 5-CSRTT (van Gaalen et al., 2006, Eagle et al., 2008, Navarra et al., 2008). Globally reducing forebrain 5-HT, increased premature responding on the 5-CSRTT (Harrison et al., 1997) and disrupted acquisition and performance of a go/no-go task (Harrison et al., 1999). However increased 5-HT levels in the PFC were associated with elevated premature responding in the “one choice” task (a simplified version of the 5-CSRTT) (Dalley et al., 2002). Therefore, it has been suggested that global reduction in 5-HT increases impulsive action, whereas reduction in 5-HT in specific receptors or brain regions decreases impulsive action (Talpos et al., 2006).

As well as the known interplay between DA and 5-HT, NA has also been implicated in the modulation of impulsivity (Economidou et al., 2012). Dysregulation of NA neurotransmission has been widely linked with the manifestation of maladaptive impulsive behaviour, such as ADHD (Arnsten and Pliszka, 2011). The role of NA in impulsivity has been principally focused on motor impulsivity, with widespread findings from pharmacology studies showing that increased NA release, via injections of the NA re-uptake inhibitor, atomoxetine, reduced impulsive behaviour (Robinson et al., 2008, Winstanley, 2011). Moreover the clinical efficacy of atomoxetine as a treatment for ADHD strongly implicates NA as a modulator of impulsivity (Del Campo et al., 2013). Noradrenergic transmission has also been found to be deficient in patients with impulse control disorders (Faraone et al., 2005).
The neuroanatomical correlates of impulsive action are still not completely determined, but research has principally focused on response inhibition. Growing research has led to an increased understanding of this circuitry in humans (Kim and Lee, 2010) and pre-clinical research has assisted the identification of this circuitry in rodents (Dalley et al., 2011). Primarily the prefrontal cortical, striatal and limbic brain regions have been found to play an important role in impulsivity, with research implicating that the NAcB plays a significant role in impulsive behaviour (Dalley et al., 2011). Specifically, the PFC in conjunction with the NAcB, assists in the structuring of impulsive behaviour (Cardinal et al., 2001b, Mobini et al., 2002, Winstanley et al., 2004b). As is the case with impulsive choice, the LC has also been implicated in the modulation of impulsive action, whereby deficits in Cingulate Cotex (Cgl)-LC interactions may contribute to impaired impulse control measured by the 5-CSRTT (Dalley et al., 2008). It is suggested that glutamatergic inputs from the amygdala, hippocampus, midline thalamus and PFC, along with dopaminergic inputs from the mesolimbic dopaminergic system act together to exert an influence on the NAcB core and shell sub-regions (Caprioli et al., 2014). Recent research utilising the SSRTT in rats has shown that different facets of impulsive behaviour are governed by dissociable neural circuitry, namely that ‘waiting impulsivity’ and ‘stopping impulsivity’ depend upon entirely separate neurological systems governed by different regions of the brain (Dalley et al., 2011). It is therefore becoming increasingly clear that even within the sub-category of impulsive action, separate elements of impulsive behaviour may be neurally dissociable.

1.3.3 Summary

Impulsivity and risk-taking represent behaviours that are clinical components of a multitude of psychiatric illnesses, and are especially important in addictive disorders. Both clinical and preclinical research surrounding the neurobiology of impulsivity and risk-taking has established a role for 5-HT, DA and NA in mediating these behaviours. This has been demonstrated through a number of human and animal studies, which have utilised lesioning and drug studies to discover the specific processes involved in impulsive action, impulsive choice and risk-taking behaviour. The ways in which these neurotransmitters exert influence on certain brain regions, as well as the way they interact with other monoamine
neurotransmitters is still not conclusive. What is clear however, is that impulsivity is made up of several different dimensions, most broadly ‘impulsive choice’ and ‘impulsive action’, which are dissociable behaviourally. Although the regions of the brain controlling these behaviours are overlapping, impulsive choice and impulsive action are also dissociable neurologically. Furthermore risk-taking is an over-lapping construct with impulsive choice, where the analysis of options to make a decision is central to both of these behaviours. However, in certain circumstance (e.g. discounting paradigms) one would expect risk-taking and impulsive choice to dissociate; whereby preference of the small reward could be interpreted as both increased impulsivity and decreased risk-taking (Winstanley et al., 2004, Kalenscher, 2007).

In relation to the central aims of this thesis, mice lacking functional copies of the imprinted genes Nesp and Grb10 show behavioural phenotypes that are indicative of, and have been interpreted as altered risk-taking behaviour (Plagge et al., 2005, Garfield et al., 2011). Specifically this has been demonstrated in tasks that measure reaction to novel environments and social dominance, in Nesp<sup>+/m</sup> and Grb10<sup>-p</sup> mice respectively. Furthermore, the initial findings in Nesp<sup>+/m</sup> mice have been followed up with a study of delay discounting, with the mutant animals having a reduced tolerance of delay (Isles et al., unpublished data). Finally, many of the regions in which Nesp and Grb10 are co-expressed such as the VTA, LC and DRN, together with their forebrain projections, are central to the control of impulsivity and risk-taking. Taken together, there is a strong hypothesis for a role for these two imprinted genes in mediating impulsive/risk-taking behaviour; based on the regional distribution and expression patterns of these genes in these particular brain regions. This suggests that testing mice null for Nesp and Grb10 in behavioural tasks of impulsivity might determine if these imprinted genes influence impulsivity and risk-taking. This tentative finding highlights the need for further investigation into the role of Nesp and Grb10 in risk-taking behaviour, and therefore the need to measure risk-taking using more sophisticated behavioural paradigms than previous studies. As well as existing paradigms of risk-taking behaviour, it is also clear there is a need for the development of risk-taking tasks which have increased face validity and are more ethobiologically relevant to rodents.

By assessing, in these tasks, mice carrying specific gene deletions or transgenes, there is the possibility of further delineating the genetic contribution underlying the
neurobiology of impulsivity and risk-taking behaviours. Importantly, in combination with previous studies investigating specific brain lesioning and psychopharmacological manipulations, further insight into the neurological underpinnings of this behaviour will be reached; which in turn could lead to innovation and development of novel drug therapies for the psychiatric disorders in which abnormal impulsivity is a feature.

1.4 Aims of thesis

The genes *Nesp* and *Grb10* represent brain expressed imprinted genes that have been partially characterised behaviourally. This includes evidence for the role of *Nesp* in reactivity to novel environments and in delay-discounting behaviour; and the role of *Grb10* in social dominance. These earlier findings suggest that *Nesp* is involved in impulsive and/or risk-taking behaviours and that *Grb10*, although less well-characterised, may be involved in these behaviours also. The strikingly similar expression of *Nesp* and *Grb10* in the brain has lead to the exciting possibility that these two genes may be acting upon the same neurological and behavioural systems (Dent and Isles, 2013). Furthermore the fact that these genes are oppositely imprinted in the brain (*Nesp* – maternal expression, *Grb10* – paternal expression) has led to the hypothesis that these genes may have antagonistic roles in mediating behaviour (Plagge et al., 2005). The distinct brain regions where *Nesp* and *Grb10* are co-expressed represent areas key to the mediation of impulsivity and risk-taking. The aim of this thesis is, therefore, to functionally examine the roles of *Nesp* and *Grb10* in a variety of tests that are indicative of risk-taking and/or impulsive behaviour, and to carry out experiments to better understand the expression of these two imprinted genes. The main hypothesis of this research is that *Nesp* and *Grb10* both influence risk-taking and/or impulsive behaviour. The initial chapters are concerned with examining the behaviour of *Grb10*+ mice in those tests on which *Nesp*+ mice have previously been assessed. This is followed by a number of experiments examining behaviour of both mouse lines in a novel risk-taking task.
Specific experimental aims of the thesis are to:

- Assess $Grb10^{+/p}$ in the tests of novelty exploration and locomotor activity previously carried out with $Nesp^{m/+}$ mice (Chapter 3).
- Assess $Grb10^{+/p}$ mice on the delayed reinforcement task, to examine if $Grb10$ affects delay-discounting behaviour (Chapter 4).
- Develop a novel test of risk-taking behaviour (Predator Odour Risk Taking - PORT) (Chapter 5).
- Assess risk-taking behaviour in $Nesp^{m/+}$ and $Grb10^{+/p}$ mice using the PORT task (Chapter 6).
- Determine if Nesp55 and Grb10 are co-localised within the same cells; and examine if like Grb10, Nesp55 is co-localised with key neurotransmitters (Chapter 7).
Chapter 2 – General methods and materials

2.1 Introduction

The purpose of this chapter is to describe the general procedures that were performed throughout the course of this thesis, and illustrations of the behavioural apparatus that were employed. All procedures that involved the usage of live animal subjects were carried out in accordance with the requirements of the U.K. Animals (Scientific Procedures) Act (1986) and in line with the Home Office Project Licence granted to Dr Anthony Isles (PPL: 30/2673). Work was carried out under the Home Office Personal Licence granted to Claire Dent (PIL: 30/9361).

2.2 Subjects and animal husbandry

2.2.1 Subjects

2.2.1.1 $Nesp^{m/+}$

Nesp55 deficient mice were created as described previously in Plagge et al. (2005) using a mutation which targets the $Nesp$ coding exon, this mutation stops protein translation but has no effect on transcription. This was achieved through a lox-P flanked targeting cassette which was excised through germ-line specific expression of Cre recombinase in male chimeras, resulting in offspring carrying a mutant allele in which one lox-P site replaced the $Nesp$ translation initiation sequence. $Nesp$ is preferentially expressed from the maternal allele, therefore maternal transmission of the mutation results in Nesp55-deficient progeny, which will be referred to throughout this thesis as $Nesp^{m/+}$. These subjects were compared to wild-type (WT) littermate controls. Paternal transmission of the mutation results in deletion of the paternal allele ($Nesp^{+/p}$), which means the normally expressed maternal $Nesp$ is still intact, and is therefore functionally the same as a WT.
2.2.1.2 Grb10\(^{+/p}\)

Grb10 deficient mice were created using a LacZ:neomycin gene-trap cassette within Grb10 exon 8, as described previously in Garfield et al. (2011). Grb10 shows a complex pattern of monoallelic expression. Paternal Grb10 is transcriptionally silent and only the maternal allele is expressed in peripheral tissues; in contrast, paternal Grb10 is expressed in the CNS, where the maternal allele is silenced. Therefore, paternal transmission of the targeted mutation, referred to as Grb10\(^{+/p}\), results in loss of Grb10 expression in the CNS only. Grb10 KO animals used in this research were paternal KO of Grb10 (Grb10\(^{+/p}\)) and were compared to WT littermate controls.

Breeding stock of both lines were maintained in-house on a mixed “F1” (CBA/Ca x C57Bl/6) background. Experimental cohorts of Nesp\(^{m/+}\) plus WT littermates, and Grb10\(^{+/p}\) plus WT littermates were generated from these breeding stocks by crossing F1(CBA/Ca x C57Bl/6) with either F1 Nesp\(^{m/+}\) or F1 Grb10\(^{+/p}\) respectively to create experimental cohorts of F2 animals.

2.2.2 Animal husbandry

Mice were single-sex group housed (2-5 mice per cage) in environmentally enriched cages (i.e. with cardboard tubes, shred-mats, tissue paper) in a temperature and humidity controlled animal holding room (21 ± 2°C and 50 ± 10%, respectively) with a 12-hour light-dark cycle (lights on at 07:00 hours/lights off at 19:00 hours). Only subjects of the same line were housed together, and the majority of the home cages included at least one animal of each genotype; birth litters were kept together whenever possible. Standard rodent laboratory chow and water were available ad libitum, unless otherwise stated. Home cages were cleaned and changed once a week, at approximately the same time of the day and on the same day of the week, in order to cause minimal disruption to the behavioural testing. Only male subjects were used in all experiments in this thesis, in order to avoid the potential confound of hormonal changes in female mice. Experimental animals were regularly monitored and weighed from approximately 8-10 weeks of age for signs of ill health. Any mice showing signs of illness were immediately assessed by a Veterinarian and, if necessary, withdrawn from the experiment.
2.3 Behavioural methods

2.3.1 Handling

All behavioural cohorts were handled regularly from approximately 8-10 weeks of age, for approximately 1 minute per mouse, twice a week. Prior to behavioural testing, animals were handled every day for a period of two weeks.

2.3.2 Measurement of body weight

Body weights of all mice were recorded on a regular basis as an index of growth and development and a measure of general health. Weights were registered daily for the period of two weeks prior to and during the administration of the water restriction protocol (see section 2.3.4). This was carried out at the same time of day (at approximately 10:00). Subjects were also weighed before any behavioural experiments began.

2.3.3 Behavioural testing environment

Behavioural testing was carried out in sealed and air-conditioned testing rooms during the light period. Testing rooms were lit by fluorescent lights, except where stated. Temperature and humidity levels were not strictly controlled, but were generally maintained at around 21 ± 2°C and 50 ± 10% respectively. Rooms were thoroughly cleaned once a week.

2.3.4 Protocol for the water restriction schedule

Prior to testing in behavioural paradigms where condensed milk was used as a reinforcer, subjects were placed on a schedule of reduced home cage water access (in order to enhance motivation for the reinforcer). This schedule lasted 6 days, encompassing 4 days of 4hr access/day and 2 days of 2 hr access/day. Mice were weighed daily during this time, as were their drinking bottles to ensure they were drinking during the period of water access. In the event of an animal losing >20% of its ad libitum body weight or showing
clinical symptoms of dehydration or ill-health animals would immediately be given *ad libitum* water access until normal *ad libitum* body weight was re-established. However this was never observed. Subjects were allowed *ad libitum* access to standard laboratory chow, throughout the duration of the water restriction schedule. After these 6 days, mice were maintained on a 2 hr access/day schedule for the duration of the experiments that required water restriction. During periods of water restriction, subjects were given *ab libitum* access to water at weekends.

### 2.3.5 Reinforcer preference test/reactivity to a novel food substance

Prior to experimental testing, all subjects were habituated to the liquid reinforcer (10% condensed milk solution, Nestle Ltd, U.K.) used in behavioural tasks such as the delayed discounting task, progressive ratio task and the predator odour risk-taking (PORT) task. Briefly, the reinforcer preference test (see Plagge et al., 2005) was carried out in a number of holding cages (285 x 130 x 120 mm) with a single subject per cage, during a single 10 minute session per day, across a six-day period. During the first session, subjects were allowed to habituate to the test apparatus, while general water consumption was measured by placing two containers (max vol. 3 ml each) containing an excess of tap water of registered weight, to the rear of each cage. Following each test session, the containers were re-weighed in order to determine the total water consumption. Over the next four sessions, one of the containers was filled with the condensed milk reinforcer, and the second container was filled with tap water. The locations of the two containers within the cage were pseudo-randomly switched between days. As before, the containers were weighed prior to, and immediately after testing, in order to determine the consumption of each liquid as well as the daily preference for the condensed milk. Reinforcer preference was defined as the amount of reinforcer consumed in the final session of reinforcer preference testing, as a percentage of the total amount of liquid (i.e. the reinforcer and water collectively) consumed during that session. The final day of testing involved filling both containers with condensed milk, for the ‘milk vs. milk’ condition. This was to ensure that all of the subjects had sampled the condensed milk reinforcer before testing in the behavioural paradigms. Any subjects failing to reach 70% preference for the condensed milk on the final
day of preference, and failing to consume condensed milk on the milk vs. milk day, were excluded from any experiments using condensed milk as a reinforcer.

2.3.6 Behavioural phenotyping: general experimental control measures

Behavioural testing always coincided with the light phase of the light-dark cycle of the animal holding room (i.e. between 07:00 hours and 19:00 hours), and water access was provided immediately subsequent to any behavioural assessment. Moreover, in order to maintain a constant time period between the time of testing and water access (which might affect motivation for the liquid reinforcer), and also to minimise any behavioural variation due to when the subjects were tested, individual subjects were tested at the same time each day. In addition, possible ‘order of experimental run’ effects were negated by running the experimental and control subjects in a pseudo-random order. To minimise possible confounds related to cage/litters, the experimental subjects were drawn from as large a number of cages/litters as available.

2.3.7 Culling protocol

At the end of the experiment, or in case of illness/injury, subjects were generally culled through cervical dislocation in accordance with Schedule 1 of Animals (Scientific Procedures) Act (1986). However, for collection of brain tissue samples, a number of animals were subjected to terminal anaesthesia and perfusion with 10% formalin (outlined in Section 2.5.3.1).
2.4 Behavioural apparatus

The current section describes the apparatus used for behavioural experiments employed in this thesis. The exact procedures used are described in detail in the subsequent experimental chapters.

2.4.1 Locomotor activity

*Figure 2.1 Locomotor activity apparatus.* Testing of locomotor activity (LMA) was carried out in an apparatus consisting of twelve clear Perspex chambers (each 21 x 36 x 20 cm, width x length x height), with two embedded infra-red beams crossing each cage 30 mm from each end and 1 cm from the floor. Beam breaks were recorded as an index of activity, using a computer running custom written BBC Basic V6 programmes (Dr Trevor Humby) with additional interfacing by ARACHNID (Cambridge Cognition Ltd, Cambridge, U.K.). Data stored were the total number of beam-breaks from each 2 hour session, as well as the number of beam-breaks made over each 30 minute quartile.

See Chapter 3 for experimental details.
2.4.2 Novelty Place preference

*Figure 2.2 Novelty place preference apparatus.* Testing of novelty place preference (NPP) was carried out in an apparatus consisting of two adjacent chambers (each 30 x 30 x 20 cm, width x length x height) with an opening in the middle, which could be occluded by a door (attached with Velcro). The 2 arenas were made distinct by the colour (black or white) and the texture of the floor (plastic or sandpaper). The time spent and the number of entries into the novel compartment was measured automatically by a video tracking system, using Noldus software.

See Chapter 3 for experimental details.
2.4.3 Delayed reinforcement and progressive ratio

![Diagram of the apparatus](image)

1. Response array (9 apertures, 10 mm diameter, spanned by vertical infra-red beams and each containing a small light)
2. Food magazine (containing tray light and food well) accessed via a hinged panel
3. Silicone tubing
4. Peristaltic pump
5. Reinforcer bottle (10% condensed milk solution)
6. House light
7. Loudspeakers

Figure 2.3 Delayed reinforcement and progressive ratio apparatus. Testing was carried out in “9-hole” operant chambers (Cambridge Cognition Ltd, U.K.) based on a design first used in rats (Carli et al., 1983) and tailored for use in mice (Humby et al., 1999). The chambers were made of aluminium, with a clear Perspex roof, and a curved rear wall (at a distance of 130 mm) into which nine 10 mm diameter holes were set. Set into each aperture was a 2.5 W bulb and for the progressive ratio task, aperture 5 (from the left side) was used, and for the delayed reinforcement apertures 3 and 5 were used. Responses made by subjects into each aperture were measured by a vertically orientated infra-red beam. Opposite the aperture array was a food magazine (accessed through a clear Perspex door), into which condensed milk reward was delivered to a small food well in the floor via 0.8 mm silicone tubing (Watson & Marlowe, U.K). This food delivery process was controlled by a peristaltic pump, with standard food delivery of 22 μl (equivalent to driving the pump for 1 second). A micro-switch recorded when the door to the food magazine was opened, and two infra-red beams (located 5 mm off the floor, emanating from the side wall) detected general movement in the chambers. A loudspeaker was also located in the side wall, as well as two infra-red LEDs (providing background illumination for the CCTV systems) and two 2.5 W house lights. The chamber was located in a sound attenuating outer box complete with a fan to keep a constant low background noise and provide ventilation. Infra-red sensitive cameras mounted in the roof of the outer boxes meant that CCTV could be used to observe the mice (Watac WM6, Tracksys...
Ltd, U.K.), located 100 mm above the chambers. Control of the chambers was managed by an Acorn RISC-PC computer running custom-written BBC BASIC V6 programmes with further interfacing by ARACHNID (CeNeS Ltd, U.K.).

See Chapter 4 (delayed reinforcement and progressive ratio tasks) for experimental details.

### 2.4.4 Elevated plus maze

*Figure 2.4 Elevated plus-maze apparatus.* The elevated plus-maze (EPM) was constructed of dulled black Perspex covered in white tape (to enable tracking of dark coated mice) and consisted of two exposed open arms (17.5 x 7.8 mm, length x width) and two enclosed arms (19 x 8 x 15 cm, length x width x height) with an open roof. The maze was positioned 94 cm above the floor and illuminated evenly with a 60 W bulb.

See Chapter 5 for experimental details.
2.4.5 Startle chamber

Figure 2.5 Startle/Prepulse inhibition apparatus.
The startle chamber apparatus consisted of ventilated and sound attenuating SR-LAB startle chambers (San Diego Instruments, CA, USA) containing a non-restrictive Plexiglas cylinder (3.5 cm in diameter) mounted on a Perspex plinth. Directly beneath the centre of the tube was a Piezoelectric sensor that detected flexion in the plinth: the measure of startle reactivity. Above the animal enclosure (12 cm) a loud speaker was mounted, via which all white-noise stimuli were presented. The motor responses of the subject were recorded via the piezoelectric accelerometer and values were transduced and digitised by a transducer linked to the computer. For each day of testing, the test chambers were calibrated, using mice of equivalent bodyweight to the test subject to equalise the measure of startle response from each chamber. Similarly, the peak amplitude of the stimuli presented in each chamber was made equivalent.

See Chapter 5 for experimental details.
2.4.6 *Predator odour risk-taking task*

![Diagram of the apparatus](image)

*Figure 2.6 Predator odour risk-taking task (PORT) testing apparatus.* Apparatus consisted of three adjacent chambers of equal size (30 x 30 x 40 cm), separated by walls containing guillotine doors operated by a pulley system. A video camera, connected to a video recorder, was mounted above the apparatus to allow video tracking (by EthoVision, Noldus Information Technology, The Netherlands) of the animals behaviour and video recording of the experiments. Mice would start in one of the outer chambers (shown here as the left chamber, but counterbalanced across subjects) and would be required to cross the middle chamber (containing an odour) in order to collect a food reward in the opposite chamber (shown here as the right chamber).

See Chapters 5 and 6 for experimental details.

2.4.7 *Ethovision tracking*

Assays using the EthoVision Observer video tracking software (version 3.0.15, Noldus Information Technology, Netherlands) included the NPP task, EPM and the PORT task. Video tracking was used to track and analyse the behaviour, movement and activity of the mice. EthoVision tracks a subject’s movement within previously defined zones within the total arena, which are designed specifically for each task. The EthoVision tracking system performed calculations over a series of frames (12 frames/sec) to derive a set of quantitative descriptors about the movement and location of subjects, and variables such as duration, movement, entries and latency to enter each the chamber were determined by the EthoVision programme in terms of location of the greater body-proportion of subjects. Tracking of the subject was calibrated for the test apparatus prior to testing using non-experimental mice of the same body size and coat colour as the experimental subjects.
Specific zone designs as well as specific parameters measured are outlined in each experimental chapter.

2.5 Molecular methods

2.5.1 Standard genotyping protocol

To identify the different genotypes of mice, DNA was extracted from hair samples and/or tail biopsies (~4 mm) collected when the mice were 5 weeks old. All genotypes were re-confirmed from tail biopsy samples following culling at the end of an experiment. DNA was amplified by Polymerase chain reaction (PCR) and imaged using gel electrophoresis.

2.5.1.1 DNA extraction from hair samples

Tufts of hair were obtained by plucking with tweezers, and placed in 1.5 ml micro-centrifuge tubes. 100 μl of 50 mM NaOH (sodium hydroxide) was added to samples and centrifuged for 60 seconds at 13,000 rpm. Samples were then put on a heating block and left to heat at 100°C for 10 minutes. Following this, samples were put on ice and allowed to cool for 5 minutes, and then centrifuged for 1 minute at 13,000 rpm. 2 μl of sample was used per PCR reaction.

2.5.1.2 DNA extraction from tail samples

Tail biopsy samples were placed in 1.5 ml micro-centrifuge tubes and digested in 400 μl of lysis buffer (0.2% SDS, 50 mM Tris (tris(hydroxymethyl)aminomethane); pH=8.0, 10 mM EDTA (ethylene-diaminetetra-acetic acid), 100 mM NaCl (sodium chloride) with 2 μl of Proteinase K (Qiagen, Crawley, U.K.) with a final concentration of 0.2 mg/ml, at 55-60°C overnight. The following day, digested tissue samples were vortexed briefly and spun in a centrifuge for 10 minutes (13,000 rpm) to sediment debris, the supernatant subsequently transferred into new micro-centrifuge tubes. Next, an equal volume of cold isopropanol (400 μl) was added to the obtained supernatant and the samples refrigerated for 20 minutes to facilitate DNA precipitation. Samples were then re-centrifuged for 10 minutes (13,000 rpm), following which the supernatant was discarded and the tubes left on a
heating block for 10 minutes in order to allow the isopropanol to evaporate. A volume of 100 µl of TE buffer, (10 mM of Tris and 1 mM of EDTA, bought to pH=8 with hydrogen chloride to down-regulate the activity of nucleases), was added to stabilize and protect the dissolving DNA from degradation.

2.5.1.4 Genotyping primers

PCR genotyping was carried out using primers designed specific to Nesp, as shown in Table 2.1. Primers for Grb10 were designed to the β-galactosidase/neomycin (β-geo) cassette integrated into the Grb10 locus, the details of which can be found in Table 2.1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nesp</td>
<td>Forward (NL3)</td>
<td>AGTGGAGGCACCTCTCGGA</td>
</tr>
<tr>
<td></td>
<td>Reverse (Ne-R7)</td>
<td>TCGTGATCAGACTCAGATTCA</td>
</tr>
<tr>
<td>Grb10</td>
<td>Forward (β-geo F2)</td>
<td>CCGACGAAAAACGCTGCG</td>
</tr>
<tr>
<td></td>
<td>Reverse (β-geo R2)</td>
<td>CTTCCGCTTCAGTGACAACG</td>
</tr>
</tbody>
</table>

Table 2.1: List of primer sets used for genotyping.

2.5.1.5 Polymerase chain reaction (PCR) protocol

Reaction mixtures and the programme used for Nesp and Grb10 genotyping PCR are described in Table 2.2, using the respective primer sets detailed in Table 2.1. The reactions were run in a Peltier Thermal Cycler (MJ Research, U.K.).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Programme</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 µl of 10x buffer</td>
<td>1. 95°C for 10 minutes</td>
</tr>
<tr>
<td>2.5 µl dNTPs</td>
<td>2. 95°C for 30 seconds</td>
</tr>
<tr>
<td>1 µl Forward primer</td>
<td>3. 58°C for 30 seconds</td>
</tr>
<tr>
<td>1 µl Reverse primer</td>
<td>4. 72°C for 45 seconds</td>
</tr>
<tr>
<td>0.4 µl Taq</td>
<td>5. Stages 2-4, 30 cycles</td>
</tr>
<tr>
<td>1 µl sample</td>
<td>6. 72°C for 10 minutes</td>
</tr>
<tr>
<td>16.6 µl H2O</td>
<td>7. 10°C forever</td>
</tr>
<tr>
<td>Total: 25 µl</td>
<td>1. 96°C for 10 minutes</td>
</tr>
<tr>
<td></td>
<td>2. 96°C for 30 seconds</td>
</tr>
<tr>
<td></td>
<td>3. 60°C for 30 seconds</td>
</tr>
<tr>
<td></td>
<td>4. 68°C for 30 seconds</td>
</tr>
<tr>
<td></td>
<td>5. Stages 2-4, 36 cycles</td>
</tr>
<tr>
<td></td>
<td>6. 72°C for 300 seconds</td>
</tr>
<tr>
<td></td>
<td>7. 10°C forever</td>
</tr>
</tbody>
</table>

Table 2.2: List of reaction conditions and programme used for PCR
2.5.1.6 Gel electrophoresis

Amplicons were run on an electrophoresis gel to separate out fragment sized DNA, where 5 μl of 6x DNA loading buffer was added to each reaction product and a total of 30 μl of the reaction/loading buffer mixture was loaded on to a 1.2 % agarose gel. Finally, 5 μl of DNA ladder (Hyperladder IV, Bioline, U.K.) was loaded in the first well, and the gel subsequently run at 110 V for 30-40 minutes. After electrophoresis, the agarose gel was placed under UV light and the amplicons examined for bands. In genotyping the Nesp cohort an upper band (700 bp) corresponded to the mutant allele, a lower band (600 bp) to the WT. Therefore, the presence of 2 bands was indicative of a Nesp<sup>m/+</sup> genotype, and the presence of 1 band was indicative of a WT animal (Figure 2.6a). In the Grb10 cohort the presence of a mutant allele was identified by a single ~600 bp product, WT mice had no product present (Figure 2.6b).

![Image of DNA bands](image.png)

**Figure 2.6 Example images of DNA bands used to identify genotypes of individual animals.** (a) Shows image of genotyping for mice in the Nesp cohort, where a double band (at 600 and 700 bp) is indicative of a Nesp<sup>m/+</sup> subject, and a single band (at 600 bp) is indicative of a WT subject. (b) Shows image of genotyping for mice in the Grb10 cohort, where a single band (at ~600 bp) is indicative of a Grb10<sup>+/-</sup> subject and no band is indicative of a WT subject.
2.5.2 Real time qPCR

2.5.2.1 Preparation of brain homogenates for real-time qPCR analysis

The isolation of good quality total RNA is of paramount importance in order to produce high quality gene expression data. All usual precautionary measures were taken to prevent degradation and/or contamination of the RNA samples, i.e. tissue samples kept at -80°C immediately following dissection, and using RNA-free working surfaces and consumables.

2.5.2.2 Isolation of total RNA: Trizol method

Cerebral tissue (only hypothalamus and midbrain were processed for the current research, see Chapter 7) extracted from whole brains was transferred to lysing matrix tubes (FastPrep-24, MB Biomedicals, U.S.). 400 μl of Trizol (Tri Reagent from Sigma-Aldrich, U.K.) was added to each tube and the tubes then homogenised at 4°C in a FastPrep FP120 micro-homogenizer (MB Biomedicals, U.S.) for 2 x 10 seconds, at 5 m/s. Tubes were then spun in a centrifuge at 12,000 rpm at 4°C for 10 minutes, the supernatant removed from the matrix tubes and discarded, and 200 μl of chloroform added to each tube. The tubes were vortexed for 15 seconds and then incubated at room temperature for 10-15 minutes. The mixture was then spun in a centrifuge at 12,000 rpm at 4°C for 10 minutes, and supernatant (containing the RNA) transferred to a fresh polypropylene centrifuge tube. A volume of 300 μl of isopropanol was then added to each sample; they were then briefly vortexed (10 seconds) and then incubated at room temperature for 10 minutes. The mixture was then spun in a centrifuge at 12,000 rpm at 4°C for 10 minutes, forming a pellet; after which the supernatant was removed and discarded. Subsequently, the RNA pellet was washed in 500 μl of 75% ethanol, vortexed briefly and spun in a centrifuge at 12,000 rpm at 4°C for 5 minutes. The RNA pellet was next allowed to dry for approximately 45 minutes and then resuspended in an appropriate volume of DEPC-treated MilliQ water. Finally, the samples were incubated at 55°C for approximately 20 minutes to ensure total re-suspension.
2.5.2.3 Quantification of the RNA sample

A spectrophotometer (NanoDrop® ND-1000 UV-Vis, Wilmington, DE) was used to quantify the amount of RNA in each sample, by measurements of UV absorption. Since RNA absorbs maximally at 260 nm, the ratio of absorbance at 260 nm and 280 nm (protein) was used to assay the RNA purity of a given RNA preparation. Pure RNA has an A260/A280 ratio of 2.1. For nucleic acid quantification, the Beer-Lambert equation is manipulated to give:

\[ c = \frac{A \times e}{b} \]

Where \( c \) is the nucleic acid concentration in ng/\( \mu l \), \( A \) is the absorbance in AU (for the arbitrary absorbance units), \( e \) is the wavelength-dependent extinction coefficient in ng-cm/microliter and \( b \) is the path length in cm. For the nucleic acid, data are normalised to a 1 cm path. The generally accepted extinction coefficient for RNA is 40. The spectrophotometer enabled highly accurate analysis of particularly small sample volumes. Surface tension was used to hold a column of liquid sample in place while a measurement was made. A small quantity of the sample (1 \( \mu l \)) was pipetted directly onto a measurement pedestal, and a measurement column was then drawn between the ends of two optical fibres in order to establish a measurement path. The measurement was carried out and displayed on the screen of an attached computer.

2.5.2.4 Removal of DNA from the RNA sample

To create high quality cDNA from the RNA sample, all traces of DNA were removed from the sample using Turbo DNA-free protocol (Ambion, UK). The typical reaction was a 50 \( \mu l \) volume, containing nuclease free water, 1x TURBO DNase buffer, 1 \( \mu l \) of TURBO DNase for up to 10 \( \mu g \) of RNA. The reaction mixture was incubated at 37°C for 30 minutes. 1x DNase Inactivation reagent was then added to the reaction and thoroughly mixed. After a 2 minute incubation period at room temperature the sample was centrifuged at 10,000 rpm for 1.5 minutes. The RNA was then transferred into a fresh tube.
2.5.2.5 RNeasy purification of extracted total RNA

The RNeasy Mini Kit for RNA clean-up (Qiagen, UK) was used to purify the extracted RNA (binding capacity 100 μg) according to the manufacturer’s instructions. The total RNA sample was adjusted to 100 μl with RNase free water, and 300 μl of RLT buffer was added to the sample and mixed well. Subsequently, 250 μl of absolute ethanol was added to the RNA/RLT solution and mixed thoroughly, and then 700 μl of the sample was applied to an RNeasy mini-spin column membrane sitting in a collection tube. The sample was then spun in a centrifuge for 15 seconds at 10,000 rpm, transferred to a mini-spin column membrane of a collection tube, and spun again for 15 seconds at 10,000 rpm. Once, the RNA had been transferred into a new collection tube, 500 μl of buffer RPE was added and the solution then spun in a centrifuge for 15 seconds at 10,000 rpm to wash. The same volume of buffer RPE was added onto the column membrane again (once the remaining buffer RPE had been discarded), and then spun in a centrifuge for 2 minutes at 13,000 rpm to dry the RNeasy membrane. Next, the RNA spin column was placed in a new 2 ml collection tube and spun in a centrifuge for 1 minute. The column was then transferred into a new 1.5 ml collection tube and 20 μl of RNase-free water was pipetted directly onto the membrane and allowed to stand for 1 minute, after which it was spun in a centrifuge for 1 minute at 10,000 rpm. This step was repeated but with only 10 μl of RNase-free water this time. RNA was eluted in a total volume of 30 μl nuclease-free water and again was quantified by absorbance using the NanoDrop® ND-1000 UV-Vis Spectrophotometer machine (see Section 2.5.2.3).

2.5.2.6 cDNA synthesis

The synthesis of first-strand cDNA from the total extracted RNA was carried out using Clontech® RNA to cDNA kit for PCR (Clontech, Mountain View, U.S) in accordance with the accompanying Poly-dT protocol, based on the methods of Ausulbel et al. (1995). The extracted (and purified) total RNA in RNase-free water, was adjusted to obtain 2.5 μg of total RNA in a final volume of 20 μl, based upon the previous determination of total RNA concentration. Next, the total RNA samples were pipetted into individual wells of the Sprint RT complete product and mixed thoroughly with the lyophilised reagents by pipetting up and down. The wells containing the reaction mixtures were then incubated at 42°C for 60
minutes. Finally, the reactions were terminated by heating at 70°C for 10 minutes, thus ending the synthesis of cDNA from the RNA reactions.

2.5.2.7 Quantitative PCR (qPCR) primer sets

The primer sets used for the real-time quantitative PCR analysis, were designed using Primer3 (http://primer3plus.com/web_3.0.0/primer3web_input.htm). Each of the primer sets (Table 2.3) was designed across intron-exon boundaries, thus eliminating signals from contaminating genomic DNA. Working concentrations of primer pairs were optimised to provide the best signal-to-noise ratio. Optimisation of the primer sets was performed by combining different dilutions of the forward primer with varying dilutions of the appropriate reverse partner. Furthermore, every combination was assessed in two kinds of reactions; the real-time PCR reaction using a test DNA sample and the non-template control reactions (NTC). Since the NTC reactions did not contain any DNA sample, any false amplification signal could only originate from primer dimers. The obtained Ct values (see Section 2.5.2.9) of the NTC reactions were compared to the Ct values obtained in the DNA-containing PCR reactions. Details of the primers and the working concentrations used can be found in Table 2.3.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nesp</td>
<td>Forward</td>
<td>5'-GACGGTCAAGAAGGTGGAAA-3'</td>
<td>700 nM</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CACTGCGCCTGCAATCCC-3'</td>
<td>700 nM</td>
</tr>
<tr>
<td>Grb10</td>
<td>Forward</td>
<td>5'-TGCACCACTTTCTTGAGATG-3'</td>
<td>700 nM</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-ACCAGTGAGCTCCGGAAATG-3'</td>
<td>700 nM</td>
</tr>
<tr>
<td>18S</td>
<td>Forward</td>
<td>5'-GTAACCCGTTGAGCCCATT-3'</td>
<td>300 nM</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CCATCCAATCGGTAGAGCG-3'</td>
<td>300 nM</td>
</tr>
</tbody>
</table>

Table 2.3: List of qPCR primer sets used for the qPCR gene expression analysis.

2.5.2.8 qPCR

Gene expression analysis was performed using a Rotorgene 6000 RT-PCR machine (Corbett Research, UK), while the consumables used in the experiments were provided by Corbett Research and Bioline, UK. The reactions and programme used were set up in accordance with the protocol listed in Table 2.4, where the purpose of stage six and seven in
the programme was to obtain ‘melting curve’ data, which enabled confirmation that only a single amplicon was produced, and that minimal primer dimerisation had occurred.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Programme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4 µl of H₂O</td>
<td>1. 95°C for 10s</td>
</tr>
<tr>
<td>7.5 SensiMix 2X</td>
<td>2. 57°C for 15s</td>
</tr>
<tr>
<td>0.3 Syber (50X)</td>
<td>3. 72 °C for 20s</td>
</tr>
<tr>
<td>Forward primer</td>
<td>4. Go to Step 1; 40 times</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5. Ramp from 72°C to 95°C rising 1°C each step (melting curve analysis)</td>
</tr>
<tr>
<td>10 µl of cDNA (1/10)</td>
<td>6. Wait for 90’’ of pre-melt conditioning on first step and for 5’’ for each step afterwards.</td>
</tr>
<tr>
<td></td>
<td>7. End</td>
</tr>
</tbody>
</table>

21 µl

*Table 2.4: Protocol of reactions and programme used for qPCR analysis.*

2.5.2.9 *Data analysis using the $2^{-\Delta\Delta Ct}$ method*

Since gene expression was compared between mutant mice and their WT-littermate controls, a relative quantification method was selected as the appropriate method of analysing the data. Therefore, the $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in gene expression determined from each real-time quantitative PCR experiment. Derivation of the $2^{-\Delta\Delta Ct}$ equation, including assumptions, experimental design and validation tests have been previously described by Livak and Schmittgen (2001). Housekeeping genes are commonly used as an internal control in order to normalise the qPCR data for the amount of RNA that is added to a reaction. In this thesis, 18S was selected as the reference gene due to its widespread uniform and high levels of expression (Doe et al., 2009). The change in expression of the target gene was normalized to the Ct values of 18S rRNA for each individual sample. Each sample was run in triplicates in order to minimise sampling errors. The Ct values (defined by the number of cycles required for the fluorescence to cross the threshold, i.e. exceeding the background level) obtained from the Rotorgene 6000 RT-PCR software were imported into Microsoft Excel, which enabled descriptive analysis of the data, and conversion to $2^{-\Delta\Delta Ct}$ for subsequent analysis (see VanGuilder et al., 2008). The Ct values for the 18S and the target gene mRNAs were averaged across the triplicates for each sample, prior to performance of the $\Delta Ct$ calculation. The $\Delta Ct$ value was calculated by subtracting average Ct values of the target genes from the average Ct values of the 18S (i.e.
the control sample). Next, the subtraction of the ΔCt values of the control samples from the ΔCt values of the target gene samples yielded the ΔΔCt values. The negative values of this subtractions, the - ΔΔCt values, were then used as the exponent of 2, and represent in “corrected” number of cycles to threshold (Livak and Schmittgen, 2001), but the exponent conversion is based on the fact that the reaction doubles the amount of product per cycle. These values were then combined with the values of relative change in the expression of the target gene between the comparison groups and presented as a graph.

2.5.2.10 Statistical analysis of the real-time qPCR data

The final stage of the real-time qPCR analysis was to determine the threshold cycle or the Ct value. The Ct value was derived from a log-linear plot of the PCR signal against the cycle number, which depicts the Ct value as an exponential instead of a linear term. Hence, the data were converted to a linear form using $2^{-\Delta C_t}$ (transformed data). In order to examine the statistical significance of the relative change, independent two tailed T-tests were performed on the transformed data.

2.5.3 Histology

2.5.3.1 Adult mouse perfusion

$Grb10^{+/p}$ mutant mice, plus their WT littermates, and $Nesp^{m/+}$ mutant mice and their respective WT littermates were anaesthetised using an intraperitoneal (IP) injection of pentobarbitone. Once the mouse was fully anaesthetised (as determined by toe-pinchn), the chest was cut open and the right atrium of the heart was punctured to allow blood to drain from the circulatory system. Mice were then fixed via perfusion using PBS followed by 4% PFA in PBS, through an injection into the left ventricle of the heart. Brains were dissected whole and placed in 4% PFA overnight, the following day tissues were then transferred to a 30% sucrose solution (in PBS) at 4°C for 24 hours, in order to dehydrate and cryoprotect them.
2.5.3.2 Mictrotome sectioning of tissues

In order to complete immunohistochemistry and immunofluorescence analysis, whole brain samples were sectioned using a freezing microtome. Before sectioning, the cerebellum and olfactory bulb were removed from whole brains. Brains were then mounted on to the microtome platform using an embedding matrix and allowed to freeze fully. Serial coronal sections of 40 µm were sliced and put into 25-well containers containing cryoprotectant (6 sections per well). The free-floating sections in cryoprotectant were stored at -20°C until required.

2.5.3.3 Antibodies

The Grb10<sup>+/p</sup> mutant mouse strain is derived by insertion of a LacZ:neomycin<sup>+</sup> gene-trap cassette within Grb10 exon 8, therefore transmission of the Grb10KO allele separately through the paternal line generates heterozygous progeny in which the paternal Grb10 allele is disrupted by the β-geo cassette (Grb10<sup>+/p</sup>). Therefore, the LacZ reporter gene is expressed in place of paternal Grb10. As a proxy for Grb10 visualisation, a β-galactosidase (β-gal) specific antibody was used as previously (Garfield et al., 2011). The Nesp55 primary antibody (generated and obtained from Reiner Fischer-Colbrie Lab) has been well characterised previously. Specifically it is a rabbit anti-Nesp55 polyclonal antibody, recognizing the free terminal end (GAIPIRRH) of Nesp55 (Ischia et al., 1997) used at a 1:1000 dilution (as used previously). All antibodies were optimised (with the exception of anti-Nesp55), using 3 relevant dilutions of the antibodies (based on recommended dilution), in order to determine the optimal dilution. For immunohistochemistry, a biotinylated secondary antibody was used as part of the VECTASTAIN<sup>®</sup> Elite ABC System (Vector Laboratories, Canada). The antibodies used for immunofluorescence are detailed in Table 7.1.
<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Dilution</th>
<th>Secondary antibodies</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit polyclonal anti-Nesp55 (R. Fisher-Colbrie)</td>
<td>1:1000</td>
<td>Donkey anti-rabbit ALEXA FLUOR 568 (Life Technologies)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Goat polyclonal anti-β-gal (Abcam)</td>
<td>1:1000</td>
<td>Donkey anti-goat ALEXA FLUOR 488 (Life Technologies)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Goat polyclonal anti-Tyrosine Hydroxylase (Abcam)</td>
<td>1:500</td>
<td>Donkey anti-goat ALEXA FLUOR 488 (Life Technologies)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rat polyclonal anti-Serotonin (Abcam)</td>
<td>1:500</td>
<td>Chicken anti-rat ALEXA FLUOR 488 (Life Technologies)</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table 7.1 Details and optimized dilutions of primary and secondary antibodies used for immunofluorescence analysis.

2.6 General data presentation and statistical methods

The data in this thesis is presented as mean values ± standard error of the mean (SEM), calculated from the following formula;

\[
\text{Standard error of the mean} = \frac{\text{standard deviation of values}}{\sqrt{\text{number of values}}}
\]

All statistical analyses were carried out using SPSS (version 18.0 for windows). The data were analysed by either, 2-tailed, paired T-test, independent-samples T-test, one-way analysis of variance (ANOVA) or repeated measures analysis of variance (ANOVA), where appropriate. All significance tests were performed with an alpha value of <0.05 regarded as significant. Interaction statistics are only reported if they reach significance, unless otherwise stated in experimental chapters. Post hoc pairwise comparisons were performed using Tukey’s HSD test. Repeated measures data were assessed for equality of variance using Mauchley’s test of sphericity, where if this was significant Greenhouse-Geisser corrections were applied. Wilcoxon Signed Rank test or Kruskal-Wallis H tests were applied as a nonparametric substitute for t-test or repeated measures analysis where required. Specific details of statistical analyses are described in the relevant experimental chapter.
Chapter 3 – Investigating novelty exploration in *Grb10<sup>+/p</sup>* mice

3.1 Introduction

Previous research has found that maternally expressed Nesp55, encoded by the gene *Nesp*, has a role in mediating adult behaviour (Plagge et al., 2005). Research exploring the role of Nesp55 has employed the use of various behavioural tests in order to characterise the specific nature of the effect that Nesp55 has on behaviour. Behavioural testing utilising genetically modified animals, null for the maternally expressed protein, have indicated that *Nesp<sup>m/+</sup>* mice have specific changes in their reactivity to novel environments (Plagge et al., 2005) suggesting that Nesp55 itself may be associated with the promotion of risk-taking behaviour. Impulsivity and risk-taking are fundamental components of numerous psychiatric illnesses, such as schizophrenia, and attention deficit hyperactivity disorder (ADHD). Nesp55 is also expressed in regions that represent key areas within the monoamine neurotransmitter system which are associated with modulating risk-taking and impulsive behaviour, further strengthening the link between these behaviours and this transcript (Plagge et al., 2005).

The Locomotor activity (LMA) test provides an automatic measure of general motor activity. Assessing locomotor activity has been widely used as a behavioural assay, as unconditioned motor activity in rodents probes a variety of behaviours (Paulus et al., 1999). Although it is a relatively simple task, the constructs it measures are multifaceted. The interpretation of spontaneous locomotor activity in rodents has been varied; including arousal, novelty seeking, exploration, anxiety and stereotypy (Geyer, 1990). In the LMA task animals were placed in a novel cage and their movement was measured by infra-red beams for 2-hour sessions on three successive days. As well as using this task to quantify the spontaneous activity of an animal, we used this test as a measure of reactivity to a novel environment; and examined the way in which subjects reacted, and became habituated to a novel surrounding. Using this test, it has been previously demonstrated that *Nesp<sup>m/+</sup>* mice were more active than their wild-type (WT) counterparts, but only during the first day of the 3-day test (Plagge et al., 2005). This finding was supported by data from the open field (OF)
test, where \( Nesp^{m/+} \) were also more active, specifically performing more quadrant crossings than controls. Importantly however, the movement in the open field was not indicative of anxiety-related behaviour, as although the \( Nesp^{m/+} \) animals were more active, they spent no extra time in the centre of the open field arena, which is generally regarded as the primary measure of anxiogenic behaviour. Furthermore, behaviour in the elevated plus-maze, another test of anxiety which specifically assesses the conflict between approach and avoidance, confirmed unaltered anxiety in \( Nesp^{m/+} \) mice. Therefore, these data gave rise to the conclusion that \( Nesp^{m/+} \) mice show increased reactivity to novel environments, but independent of differences in anxiety (Plagge et al., 2005).

In addition to the measurement of reactivity to novelty in the LMA and open field tasks, the novelty place preference (NPP) test was also used, as it provides a specific test of novelty seeking behaviour (Mikaelsson et al., 2013). This paradigm assesses the conflict between the propensity to explore a novel environment and the fear provoked by the uncertainty of entry to a novel environment. During the NPP task animals were given a period of time to habituate to an environment, followed by the opportunity to explore a novel environment or remain in the familiar habituated environment. The degree to which the animal explored the novel environment can be indicative of novelty seeking behaviour (Redolat et al., 2009). An increased locomotor response to a novel environment is thought to be a marker of novelty seeking, an aspect of impulsivity/risk-taking behaviour (Beckmann et al., 2011). Previous research has demonstrated that preference for novelty, in a NPP task, predicts risk-taking behaviours, such as addiction and drug self-administration in rodents (Belin et al., 2010). It has been shown that rats with heightened locomotor response to a novel environment in a NPP task also show enhanced self-administration of amphetamine (Cain et al., 2005). In a previous study using the NPP task, it was shown that whilst \( Nesp^{m/+} \) mice made significantly more entries into the novel environment, they spent significantly less time in the novel environment than WT mice (Plagge et al., 2005). Researchers have interpreted these findings as being indicative of risk-averse behaviour (Plagge et al., 2005, Isles et al., 2006).

The altered reactivity to novel environments in addition to the attenuated exploration behaviour that was exhibited by \( Nesp^{m/+} \) mice led to the suggestion that Nesp55 normally acts to promote ‘risk-taking’ behaviour (Isles et al., 2006). However the
explanations for the underlying neural mechanisms that are driving this behaviour remain unresolved. Previous experiments analysing the expression pattern of Nesp55 have identified expression in discrete, but key, areas of the serotonergic and noradrenergic systems, for example in the dorsal raphe nucleus and locus coeruleus (Plagge et al., 2005). Lesions to these areas, as well as pharmacological manipulation of these areas have shown an impact on novelty exploration, impulsivity and risk-taking behaviours (Dalley and Roiser, 2012, Macoveanu et al., 2013). This suggests that Nesp55 may be exerting its influence on risk-taking and impulsivity via the serotonergic system. Recently it has also been shown that the paternally expressed imprinted gene Grb10 which is oppositely imprinted to Nesp gene, is expressed in very similar areas to that of Nesp (Garfield et al., 2011), suggesting that this overlapping expression may give rise to the possibility that Grb10 may be exerting an influence on similar adult behaviours as Nesp. Therefore, this Chapter outlines experimental work that addresses this hypothesis, focusing on examining reactivity to novel environments in Grb10+/p mice and their WT littermates using the LMA and NPP, under the same conditions as the previous study investigating Nesp+/m mice (Plagge et al., 2005).

3.2 Methods

3.2.1 Subjects, handling and genotyping

The subjects used in the experiments outlined in this chapter consisted of a cohort of 13 Grb10+/p mice and their 11 WT littermates. All animals were male, aged 16-20 weeks at the beginning of testing, Grb10+/p mice weighed an average of 32g (±2g) and WT mice weighed an average of 35g (±2g). Animals had ad libitum access to standard lab chow and water for the duration of the experiments, (except when they were in the experimental apparatus). Details of general housing, husbandry and handling leading up to the experimental period were as described in Chapter 2, Section 2.3.

3.2.2 Locomotor Activity

LMA was measured using specific test apparatus outlined in Chapter 2, Section 2.4.1. This consisted of a battery of 12 activity cages, each measuring 21 x 21 x 36 cm. The activity cages were clear Perspex boxes containing two transverse infrared beams 10 mm from the
floor, spaced equally along the length of the box, linked to Acorn computer using ARACHNID software (Cambridge Cognition Ltd., Cambridge, UK). The number of times an animal crossed the infrared beams was measured automatically, and the behavioural measure was obtained in terms of ‘breaks’ and ‘runs’. A ‘break’ was defined as a crossing one of either of the infrared beams, and a ‘run’ was defined as crossing both the infra-red beams consecutively. Animals were placed individually into the boxes, and were examined in the LMA test for 2 hour sessions in the dark, across 3 consecutive days. The experiment was carried out at the same time of day, and the animals were placed in the same box, for each of the 3 days. The cages were thoroughly cleaned after each animal, using 1% acetic acid solution.

3.2.3 Novelty Place Preference

NPP was assessed using the apparatus described in Chapter 2, Section 2.4.2. This consisted of two adjacent boxes that have an opening in the middle which could be occluded by a door. Each box measured 29 x 29 x 32 cm. The 2 boxes were made distinct by the colour (black or white) and the texture of the floor (smooth plastic or sandpaper). In the first stage of the test the door is closed and the animal is placed in one of either of the two boxes for 1 hour and allowed to habituate. The mouse was then removed, the door opened and the mouse put back in the habituated side. Subjects were then free to explore both the habituated side and the novel side for 30 minutes. The side in which the animal was habituated, and in which the sand-paper was placed, was pseudo-randomly allocated to avoid confounding the results.

The movement of each subject was tracked using a camera mounted approximately 2 metres above the test area, which was connected to EthoVision Observer software (Noldus Information Technology, The Netherlands). The test area was divided into 2 virtual ‘zones’, which were defined as ‘habituated’ and ‘novel’. EthoVision video tracking software was used to obtain behavioural data, as detailed in Chapter 2, Section 2.4.7. Behavioural measures, obtained automatically in 5 minute bins, included the duration of time spent in each zone, frequency of entries into the each zone, latency of first entrance into the novel zone and amount of rearing. The testing apparatus were cleaned after each animal using 1% acetic acid solution.
Chapter 3 | Investigating novelty exploration in *Grb10*/*p* mice

3.2.4 Statistics

LMA data (number of breaks and runs) were analysed using a 3-level repeated measures ANOVA, whereby the within-subjects factors were DAY (test days 1-3) or BIN (24 x 5 minute bins within a session) and the between-subjects factor was GENOTYPE (control, *Grb10*/*p*). The primary measure of NPP was the total duration, the number of entries made into the novel arena, and the latency to the first entrance into the novel arena; a student’s t-test was employed to analyse the difference in total time spent and number of entries made, and latency into the novel arena between the *Grb10*/*p* and WT mice. Where the data do not meet the assumptions of independence for parametric testing, non-parametric tests were used; a Kruskal-Wallis H test was employed to analyse the genotype difference in time spent in the habituated and novel environments. Data was also analysed by BIN for both the 1 hour ‘habituation’ stage, and 30 minute ‘test’ stage. Whereby a repeated measures ANOVA was used for habituation data, whereby within-subjects factors were BIN (6x 5 minute bins within a session) and the between-subjects factor was GENOTYPE (control, *Grb10*/*p*); where the data did not meet assumption of independence for parametric testing (during the choice stage) a Kruskal Wallis test was used to analyse data. All significance tests were performed at an alpha level of 0.05. Greenhouse-Geisser degrees of freedom (df) corrections were applied as appropriate to repeated-measures factors. Behavioural data are presented as mean values ± the standard error of the mean (SEM). All statistical analysis was carried out using SPSS (Version 18.0).

3.3 Results

3.3.1 Locomotor Activity

Results of the LMA task revealed that all subjects exhibited the expected habituation to the test boxes over the three days of testing. This was evidenced by a significant decline in activity across time, both in terms of the number of breaks (Figure 3.1a; main effect of DAY: F2,42=8.37, p<0.001) and the number of runs (Figure 3.1b; main effect of DAY: F2,42=7.70, p<0.005). Data from 1 *Grb10*/*p* subject was removed from all analysis due to increased variance (defined as 2 standard deviations away from the mean). There were no significant differences in locomotor activity between *Grb10*/*p* and WT mice, as measured by
the number of breaks (Figure 3.1a; main effect of GENOTYPE: F_{1,21}=0.47, p=0.50) or the number of runs (Figure 3.1b; main effect of GENOTYPE: F_{1,21}=0.01, p=0.95), across 3 days.

A more detailed analysis of the activity during each of the 120 minute LMA sessions was carried out in order to determine if \textit{Grb10}^{+/p} and WT mice exhibited parallel behaviour throughout the task. A repeated measures ANOVA was used to analyse the activity in 5-minute time bins, in terms of the number of breaks (Figure 3.2) and the number of runs (Figure 3.3). The analysis of the number of breaks confirmed a significant decline in activity across time bin for day 1 (Figure 3.2a; main effect of BIN: F_{23,483}=13.98, p<0.001), day 2 (Figure 3.2b; main effect of BIN: F_{23,483}=8.94, p<0.001) and day 3 (Figure 3.2c; main effect of BIN: F_{23,483}=10.02, p<0.001). This effect was mirrored in the number of runs, which showed a decline on day 1 (Figure 3.3a; main effect of BIN: F_{23,483}=15.78, p<0.001), day 2 (Figure 3.3b; main effect of BIN: F_{23,483}=10.45, p<0.001) and day 3 (Figure 3.3c; main effect of BIN: F_{23,483}=12.45, p<0.001). There was no difference between \textit{Grb10}^{+/p} and WT mice in the number of breaks for day 1 (Figure 3.2a; main effect of GENOTYPE: F_{1,21}=0.03, p=0.86), day 2 (Figure 3.2b; main effect of GENOTYPE: F_{1,21}=1.32, p=0.27) or for day 3 (Figure 3.2c; main
effect of GENOTYPE: $F_{1,21}=0.37, p=0.55$). Furthermore there was no difference between $Grb10^{+/p}$ and WT mice in the number of runs for day 1 (Figure 3.3a; main effect of GENOTYPE: $F_{1,21}=0.20, p=0.66$), day 2 (Figure 3.3b; main effect of GENOTYPE: $F_{1,21}=0.10, p=0.76$) and for day 3 (Figure 3.3c; main effect of GENOTYPE: $F_{1,21}=0.01, p=0.92$). These data show that $Grb10^{+/p}$ mice showed an unaltered reactivity to the novel environment, showing analogous behaviour to the WT mice throughout the LMA task.

![Graphs showing the number of breaks performed by $Grb10^{+/p}$ and WT mice across 3 days of the LMA.](image)

*Figure 3.2 The number of breaks performed by $Grb10^{+/p}$ and WT mice across 3 days of the LMA.* Graphs depict number of ‘breaks’, broken down into 5 minute bins for Day 1 (a) Day 2 (b) and Day 3 (c). The data presented are mean values ± SEM. ***$P<0.005$.**
Figure 3.3 The number of runs performed by Grb10<sup>+/p</sup> and WT mice across 3 days of the LMA. Graphs depict number of ‘runs’, broken down into 5 minute bins for Day 1 (a) Day 2 (b) and Day 3 (c). The data presented are mean values ± SEM. ***P<0.005.

3.3.2 Novelty Place Preference

3.3.2.1 Habituation stage

During the 1 hour habituation stage of the NPP Grb10<sup>+/p</sup> and WT mice showed similar behaviour, specifically there was no difference in the amount of distance travelled (Figure 3.4a; t=0.18 (df=21), p=0.86). Furthermore, subjects showed similar amounts of rearing, a behavioural index of novelty exploration, (Figure 3.4b; t=-0.01 (df=21), p=0.99). When the data were broken down into 5 minute bins across the 1 hour session (12 bins in total), the amount of exploratory behaviour, as measured by distance moved, significantly declined throughout the 1 hour habituation period (Figure 3.4c; main effect of BIN: $F_{11,231}=14.62$, p<.0001). There were no genotype differences in the distance travelled across bins (Figure 3.4c; main effect of GENOTYPE: $F_{1,21}=0.03$, p=0.86). The decline in exploratory behaviour
was also evidenced by a significant decline in rearing across the habituation session (Figure 3.4d; main effect of BIN: $F_{11,231}=5.17$, $p<.0001$), with no genotype difference (main effect of GENOTYPE: $F_{1,21}=0.00$, $p=0.99$). These data therefore show that $Grb10^{+/p}$ and WT had similar behaviour during this stage of the NPP, and demonstrated the expected habituation to the novel environment; evidenced by a significant decline in exploratory behaviour throughout the habituation session.

Figure 3.4 Behaviour of $Grb10^{+/p}$ and WT mice during the habituation stage of the NPP. The $Grb10^{+/p}$ mice showed analogous behaviour to the WT mice during the 1 hour habituation stage of the NPP. Specifically they showed no difference in the distance travelled (a), or in the amount of rearing (b), during the habituation period. When the data were broken down into 5 minute bins, there was a significant decline in exploratory behaviour, as evidenced by reduced movement (c) and rearing (d). ***$p<0.005$. 
3.3.2.2 Test stage

Results from the test stage of the NPP task showed that, in general, all mice had a propensity to explore the novel arena, shown by the overall distribution of behaviour for each subject (Figure 3.5). Data from one WT subject was removed from all stages of the NPP due to escaping from the apparatus during the habituation stage. A Kruskal-Wallis H test revealed all subjects spent significantly more time in the novel arena compared to the habituated arena (Figure 3.6a; $\chi^2(1)=16.08$, $p<0.001$ ). There was no difference between Grb10$^{+/p}$ and WT mice in the amount of time spent in the habituated (Figure 3.6a; $\chi^2(1)=0.55$, $p=0.46$) or novel arena (Figure 3.6a; $\chi^2(1)=0.47$, $p=0.50$), furthermore there was no difference in the latency of the first entrance into the novel arena (Figure 3.6b; $t=-0.32$ (df=21), $p=0.75$). Subjects from each group also showed similarities in the number of entries made into the novel arena (Figure 3.6c; $t=-0.549$, (df=21), $p=0.59$). These data were further analysed in 5 minute bins across the 30 minute session, a Kruskal-Wallis H test showed no significant differences between Grb10$^{+/p}$ and WT mice in the duration of time spent in the novel arena across all time bins (Figure 3.6d; $\chi^2(1)=2.241$, $p=0.13$).

Figure 3.5 Example traces of Grb10$^{+/p}$ and WT behaviour in the test stage of the NPP, taken by a video tracking system. During the test stage of the NPP, subjects were free to explore both chambers, counter-
balanced between mice of each genotype. In the example traces shown above, the novel chamber is white and the habituated chamber black for both mice. The traces show that both Grb10+/p (left) and WT (right) mice showed a propensity to explore the novel environment, as expected.

**Figure 3.6 Behaviour of Grb10+/p and WT mice in the NPP.** Both Grb10+/p and WT mice spent significantly more time exploring the novel arena compared to the habituated arena (a). There was no difference between Grb10+/p and WT mice in the latency to make the first entrance into the novel arena (b), or the total number of entrances made. The data presented are mean values ± SEM. ***P<0.005.
3.4 Discussion

The principal aim of this chapter was to assess the behavioural response of Grb10\(^+/p\) mice to novel environments, to gauge their performance in relation to a previous study with Nesp\(^{m/+}\) mice (Plagge et al., 2006). Behaviour was assessed using the tests of novelty reactivity and exploration, identical to that used previously (Plagge et al., 2006). These tests, the LMA and NPP, provided a basic characterisation of the activity of mice in novel environments; which taken together, can be used as an indicator of risk-taking and impulsive behaviour.

The LMA and the NPP tasks successfully targeted a measure of spontaneous exploratory behaviour. For the LMA, this was demonstrated by the fact that exploratory behaviour significantly reduced both across the three days of testing (Figure 3.1) and within each of the 2 hour sessions, both in terms of breaks and runs (Figure 3.2 and Figure 3.3, respectively). This behaviour is indicative of habituation to the novel environment, with the mice exploring the apparatus less over time. Furthermore, mice of both genotypes showed typical exploratory behaviour in the habituation phase of the NPP task. All of the subjects were initially inquisitive about the novel chamber and displayed high levels of movement and rearing in this environment, and by the end of the session, the mice displayed a significant decline in their explorative activity as they became habituated to the environment and it became familiar (Figure 3.4c and 3.4d).

The results of the LMA demonstrated that Grb10\(^+/p\) mice have analogous behaviour to that of their WT littermate mice. Grb10\(^+/p\) mice carried out an equivalent amount of ‘breaks’ and ‘runs’ on all three days of testing in the LMA task. The results found here are consistent with previous results (Garfield et al., 2011) that have measured locomotor activity using slightly different behavioural assays, which found no dissociation between Grb10\(^+/p\) and WT animals. This suggests that Grb10 does not influence levels of motor activity or novelty reactivity in this task.

The NPP task provided a more specific test of novelty exploration, as it explicitly measured novelty-investigation/seeking behaviour, by measuring the preference for a novel environment compared to a familiar environment. The results showed that all subjects spent significantly more time in the novel environment compared to the habituated
environment, as expected (Figure 3.6a); thus confirming that mice have a natural propensity to explore a novel environment (Figure 3.5). This is consistent with previous research employing the use of NPP, and is considered normal reactivity to a novel environment (Plagge et al., 2005, Milkaelsson et al., 2013). There was no significant difference between $Grb10^{+/p}$ and WT littermate animals in terms of the number of entries into the novel arena or time spent in the novel arena, which suggests that $Grb10^{+/p}$ mice do not have altered reactivity to novel environments and therefore that the $Grb10$ gene itself is not associated with the aversion or promotion of novelty-seeking behaviour in this task. Based on the fact that $Grb10$ and $Nesp$ show overlapping expression in the brain, and their opposing patterns of imprinting, it was predicted that $Grb10$ may have had the opposite effect to $Nesp^{m/+}$ mice on behavioural responses to novel environments, whereby $Grb10^{+/p}$ animals might spend significantly more time in the novel arena than WT. However the results found here are not consistent with this hypothesis.

As well as the LMA and NPP tasks, previous research assessing $Nesp$, utilised the OF test to show that $Nesp^{m/+}$ mice demonstrated increased excitement towards a novel environment; specifically performing more quadrant crossings than WT mice (Plagge et al., 2005). The OF test has also been previously carried out in $Grb10^{+/p}$ animals, the results of which indicated that $Grb10^{+/p}$ mice do not have altered behaviour in this task in comparison to WT mice (Garfield et al., 2011). The results of the previously carried out OF test, coupled with the results of the two tasks carried out in the present chapter (LMA and NPP), illustrate that $Grb10^{+/p}$ mice do not show altered novelty-seeking behaviour. This suggests that $Grb10$ may exert its influence on adult behaviour in a different way to Nesp55 and, if it is involved in impulsive or risk-taking behaviour, in a different facet of this cognitive function.

Research exploring the role of $Nesp$ has employed the use of various behavioural tests in order to characterise the specific nature of the effect that Nesp55 has on behaviour. This has included tests of LMA, novelty reactivity, as well as tests of fear and anxiety. It is using this spectrum of tests that has allowed the observation that disruption of normal $Nesp$ expression specifically affects novelty reactivity independently of general activity, and furthermore that the effects were independent of differences in fear or anxiety-related behaviour. The dissociable nature of impulsive and risk-taking behaviour means that different behavioural tasks can be used to target and measure distinct elements of
impulsivity (Dent and Isles, 2011). These can be broadly divided into two categories: tasks measuring impulsive choice or impulsive decision-making, and those that measure impulsive action or motoric impulsivity (Winstanley et al., 2004). The paradigms employed in this chapter were used to measure spontaneous novelty-seeking behaviour, an aspect of risk-taking and impulsive action; they therefore do not assess more complex types of impulsivity such as cognitive or decision-making impulsivity. Therefore, to elucidate the potential effects Grb10 may have on impulsive or risk-taking behaviour, Grb10+/p mice need to be assessed on a wider range of tasks within the umbrella of ‘impulsivity’.

3.4.1 Summary of key results from Chapter 3

- *Grb10*/p and WT littermate controls demonstrated the expected pattern of habituation behaviour in the LMA task.
- *Grb10*/p and WT littermate controls demonstrated the expected pattern of behaviour in response to a novel environment in the NPP task, showing a preference for the novel arena.
- *Grb10*/p and WT mice showed equivalent behaviour in the two assays of spontaneous novelty exploration; suggesting *Grb10*/p mice have normal levels of novelty reactivity/exploration.
Chapter 4 – Assessment of delay discounting behaviour in *Grb10*/*p* mice

4.1 Introduction

Previous research has demonstrated increased reactivity in *Nesp*/*m/+* mice when placed in a novel environment, with corroboratory evidence derived from three separate tasks: locomotor activity (LMA), novelty place preference (NPP) and open-field (OF) (Plagge et al., 2005). Additionally, as yet unpublished work has shown, maternal *Nesp* affects behaviour measured in the delayed reinforcement task, a ‘delay-discounting’ paradigm (Isles et al., unpublished research). These results have been interpreted in the context that the maternally expressed imprinted gene *Nesp* is associated with the promotion of risk-taking behaviour. Chapter 3 described the results of testing of *Grb10*/*p* mice on the LMA and NPP tests, which found that *Grb10*/*p* mice did not exhibit altered behaviour in these tests, in contrast to the pattern of behaviour shown by *Nesp*/*m/+* mice.

The ‘delay-discounting’ paradigm used in this thesis to assess behaviour in mice is based on the principle of selecting between either small immediate gratification or larger but delayed gratification. This concept was developed from the ‘Stanford Marshmallow Experiment’ (Mischel et al. 1972), which sought to investigate the age at which delayed gratification, the ability to wait for something desired, developed. In this experiment, children were presented with the choice between a single marshmallow immediately and two marshmallows at a later time. The majority of children preferred to wait for the larger reward, and the ability to tolerate longer delays increased with age. In the delay discounting task selection of the small immediate reward is considered to be indicative of impulsive choice, whilst selection of large delayed reward is considered to be indicative of increased ‘self control’ (Evenden and Ryan, 1996, Winstanley et al., 2004). Delay-discounting tasks have since been developed to measure this decision making behaviour in adults and are usually based on hypothetical or actual monetary reward (Madden et al., 2003, Demurie et al., 2012). A bias towards impulsive choice, measured in this type of task, has been observed as a symptom in several psychiatric conditions, including attention deficit...
hyperactivity disorder (ADHD) (Demurie et al., 2012), pathological gambling (Albein-Urios et al., 2012) and drug addiction (Kirby et al., 1999, Amlung et al., 2012, Kobiella et al., 2013).

The delayed-reinforcement task has also been adapted for use with animals, including pigeons (Rodriguez and Logue, 1988), macaques (Hayden and Platt, 2007), and rodent analogues for rats (Evenden and Ryan, 1996) and mice (Isles et al., 2003). Decision making behaviour measured in delay-discounting tasks has been shown to be sensitive to experimental manipulation in animals (Evenden and Ryan, 1996, Mobini et al., 2002, Cardinal, 2006). For example administration of d-amphetamine induces preference for the smaller, immediate reinforcer, thus increasing impulsive choice in both rats (Evenden and Ryan, 1996) and mice (Isles et al., 2003), conversely administration of diazepam, metergoline and atomoxetine have all been shown to significantly decrease impulsivity by increasing preference for the large-value reward across increasing delay (Evenden and Ryan, 1996, Robinson et al., 2008). Furthermore lesions to the orbital prefrontal cortex, the medial prefrontal cortex and basolateral amygdala in rats have all induced an increase in impulsive choice in tests of delay-discounting (Mobini et al., 2002, Churchwell et al., 2009). As well as animal studies, delay discounting has also been observed in a clinical population; where a bias towards impulsive choice has been observed as a symptom in several psychiatric conditions, including Attention deficit hyperactivity disorder (ADHD) (Demurie et al., 2012), pathological gambling (Albein-Urios et al., 2012) and drug addiction (Kirby et al., 1999, Amlung et al., 2012, Kobiella et al., 2013).

As mentioned previously Nesp$^{m/+}$ mice showed increased impulsive responding when tested in a delayed-reinforcement task (DRT) (Isles et al., unpublished research). During this task the delay associated with the larger reward was progressively delayed within each test session (consecutive blocks of trials with delays of 1s, 8s, 16s), whilst the small reward was consistently associated with a short (1s) delay. It was found that under increasing delay conditions both types of mice switched responding to the smaller reward associated with a fixed short delay (Figure 4.1), however as the delay increased, Nesp$^{m/+}$ mice became increasingly reluctant to wait for a reward, and instead opted for immediate gratification; thus exhibiting significantly altered delay-discounting behaviour, indicative of increased impulsive ‘choice’ behaviour.
Figure 4.1 Performance of Nespm/+ and WT control mice in the DRT. Nespm/+ mice, lacking functional Nesp55, and their WT littermates were tested in the DRT under increasing delay conditions (a) (Isles et al., unpublished results). All subjects switched their responding to the smaller rewards associated with a fixed delay (1s) as the delay to the large reward increased. However, Nespm/+ animals were quicker than WT to discount the large reward against the delay, demonstrating that these Nespm/+ mice are significantly less risk-taking than their littermates. Therefore, this suggests that the Nesp55 protein serves to promote risk-taking behaviour. As a control, when the large reward was associated with a fixed delay and remained at 1s in all trials, both Nespm/+ and WT mice maintained a preference for the large reward throughout the session (b). This demonstrates that the effect found was not as a result of satiety or fatigue affecting responding in the task. Data shown are the mean ± SEM. Control N=7, Nespm/KO N=9.

The previous finding that Nesp influences delay-discounting behaviour (Isles et al., unpublished results), taken together with the reciprocal pattern of imprinting between Nesp and Grb10 (Plagge et al., 2005, Garfield et al., 2011), and the overlapping expression of Nesp and Grb10 in areas of the brain that are involved in impulsivity/decision-based behaviour, suggests that Grb10 may also influence delay-discounting behaviour, and perhaps in an opposing direction to Nesp. Therefore, the experiments in this chapter address this hypothesis by assessing if behaviour in the DRT is altered in Grb10/+ mice compared to their WT littermates. Furthermore, as an additional control measure to dissociate DRT performance from effects on motivation, Grb10/+ mice were also assessed in a progressive ratio task (PRT).
4.2 Methods

4.2.1 Subjects

13 male \textit{Grb10}^{+/p} mice and 11 WT littermates were used. Mice were 6 months old at the start of testing (mean weight: $30.38 \pm 0.49$ g). All subjects were group housed (2 to 4 mice/cage) in environmentally enriched cages (i.e. with cardboard tubes, shred-mats and tissue paper) in a temperature and humidity controlled vivarium ($21 \pm 2^\circ$C and $50 \pm 10\%$, respectively) with a 12-hour light-dark cycle (lights on at 07:00 hours/lights off at 19:00 hours). Animals had ad libitum access to standard lab chow, but home cage water was restricted to 2 hours access per day during the period of testing (access occurred after testing). This regime maintained the subjects at ~90% of free-feeding body weight. Prior to experimental testing animals were handled daily for 2 weeks (Hurst and West, 2010), and then the water restriction schedule was gradually introduced over a further 2 weeks.

4.2.2 Water restriction and Condensed milk preference test

In order to increase motivation and performance in the DRT and PRT, subjects were placed on a schedule of water restriction. Water access was reduced gradually, in accordance with the protocol outlined in Chapter 2, Section 2.3.4. After this procedure mice were maintained on a 22 hour water restriction schedule. Prior to testing a condensed milk test (CMT) was carried out in order to ensure all subjects had a $\geq 70\%$ preference for the condensed milk reinforcer. Details of how the CMT was performed are outlined in Chapter 2, Section 2.3.5.

4.2.3 Apparatus

The DRT and PRT were performed in eight ‘9-hole’ operant chambers, as described in Chapter 2, Section 2.4.3. The control of stimuli and recording of responses were managed by an Acorn RISC-PC computer running custom-written BBC BASIC V6 software (programmed by Dr Trevor Humby) with additional interfacing by ARACHNID (CeNes Ltd., U.K.).
4.2.4 Delayed-reinforcement task

4.2.4.1 Panel Push Training

Panel push training was carried out over 6 sessions (one session per day). Each mouse was put into a chamber for a period of 20 minutes and was presented with 22 μl of reward (equating to 1s delivery time) every 30 seconds. For the initial 3 sessions the panels to the food magazines were wedged open, in order to allow a period of familiarisation, and to allow the mice to learn that the condensed milk was available in the chamber. For the remaining sessions panels were free, and subjects were therefore required to push the panels in order to gain access to the condensed milk reward. Once subjects were able to panel push successfully, they continued to the next stage of training.

4.2.4.2 Single Nose Poke Training

The initial stage of the DRT was to teach subjects to nose poke in response to a stimulus in order to receive a reward (initiate a trial). During this stage a light in the central aperture came on, nose-poking in this aperture resulted in delivery of the reward. Subjects had a limited period of time (limited hold) that the light was on for, signaling the period of time that the mouse can make the ‘nose poke’. This limited hold (LH) value was set 10 seconds for each trial. Once each mouse was successful completing a minimum of 60% of trials, they were able to progress to the next stage of training. The principle parameter measured at this stage was the number of trials initiated (out of a maximum 100 trials).

4.2.4.3 Double Nose Poke Training

The double nose poke phase involved teaching subjects to make a central nose poke, followed by a second nose poke, in response to a stimulus presented (pseudo-randomly) either left or right, in order to receive the condensed milk reward. The initial stimulus (centre light) was set to a 10 second duration, and when a mouse made a central ‘initiation’ nose poke, the second stimulus was triggered (pseudo-randomly presented either left or right) for 10 seconds. Therefore the central nose poke acted as a cue to start the trial; and to focus attention on the subsequent presentation of stimuli in lateral locations. At this stage each subject was given 40 trials per session. Once each mouse could successfully double nose poke to receive a reward, at a level of at least 70% correct, they were able to
move on to the DRT proper. The principle parameter measured at this stage was the percentage of correct responses to the second stimulus.

4.2.4.4 Delayed reinforcement testing

The DRT required the subject to first make a central nose poke to initiate a trial; then make a nose poke response, either left or right, to indicate their choice between the delivery of 50 μl reward (3s of reward delivery), or a response in the other direction resulting in the delivery of 22 μl reward (1s of reward delivery) (Figure 4.2). The large and small response directions were kept constant for each mouse but were counterbalanced between subjects and across genotypes. The task comprised of three sequential blocks of 12 trials, in block 1 either response led to the delivery of reinforcer after a 1 s delay. In blocks 2 and 3 increasing delays were introduced between the response and the delivery of the larger reinforcer, whereas the delay between response and delivery of the small reinforcer was fixed at 1 s. The length of the delay introduced in blocks 2 and 3 was increased through a series of stages (A, B, and C) until baseline stage D (Table 4.1). Trials 1 - 4 in any block were ‘forced’ information trials, whereby after the central nose poke only one of the two response options was available. The order of presentation during the forced trials was counter-balanced pseudo-randomly across subjects. This was designed to provide the subjects with prior notice of the extent of any delay associated with the larger reward response. In the next 8 trials, the animals had a choice of responding to either the right or left stimulus after the central nose poke.
Table 4.1 The delay associated with the large reward during the training stages of the DRT. The task comprised of three sequential blocks of twelve trials. During the DRT the small immediate reward was always delivered after 1s throughout the duration of the task. However the large reward was delivered after an increasing delay. In block 1 of any given session the delay associated with the large reward was always 1s. Subjects started at stage A, where the delay stayed at 1s throughout. Subjects then completed stages B-D during which the delay increased incrementally during block 2 and 3. The first four trials of any block, were ‘information trials’ which, after the central nose poke, forced a choice either left or right, to allow the subject to learn the size of the delay associated with the large reward. The final eight trials were ‘choice’ trials where the subject could select between the small immediate (1s delay) reward or the larger delayed reward.

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Figure 4.2 Schematic showing the sequence of events in the DRT. Illumination of the initiation stimulus in the central aperture of the 9 hole array, signalled the start of a trial. The subject was required to initiate a trial by making a nose poke in this location. In a choice trial, stimuli were simultaneously illuminated in the apertures to the left and right; in forced trials only a single stimulus was illuminated. Through training, the animal had learnt that one side was associated with the large reward and the other with the small reward (kept consistent for each mouse, but counterbalanced across subjects); thus presenting the animal with a choice between the stimulus associated with a small immediate reward, or a large increasingly delayed reward. The subject made a choice by nose poking at one of stimuli locations and the appropriate reward delivered. The subject is then required to make a panel push to collect the reward, located on the opposite side of the chamber. Image taken from Isles et al. 2003.

Subjects completed 5 sessions at each of the four training stages (A, B, C, D) in order to allow the opportunity to learn the task and progress through the stages gradually. Subjects were required to complete >70% of trials in order to continue to the next stage. During the task, both initial (central) and choice (left or right) stimuli were presented for 10s, and were extinguished once an animal made a nose poke. If the subject made no
central nose poke when the initial stimulus was presented, it was recorded as a ‘non-started trial’; performing an initiation central nose poke and subsequently failing to make a choice nose poke was recorded as an ‘omission’. Regardless of the outcome of the trial, a new trial was started 45 s after the presentation of the previous central stimulus. The food hopper light was illuminated when the reward was delivered, and subjects were required to panel push in order to collect the food reward from the hopper. Final baseline performance was calculated as an accumulation of their last 3 sessions at stage D. The principal measure of the DRT was the bias to choose the large reward, recorded by the number of correct responses made for the large choice as a percentage of all choice trials. In addition to this measure, other parameters included number of non-started trials, number of omitted trials, the latency to initiate a trial (start latency), the latency to make a choice (choice latency), the total number of nose pokes in a session and the total number of panel pushes in a session.

4.2.5 Progressive ratio task

4.2.5.1 Subjects

The same subjects used in the DRT were used to carry out the PRT; two subjects (1 WT and 1 Grb10+/p) were removed from the study due to illness/death. This left a cohort of 12 male Grb10+/p mice and 10 WT littermates. As before, the animals had ad libitum access to standard lab chow, and were maintained on a 22 hour water restriction schedule during the period of testing (2 hour access occurred after testing).

4.2.5.2 Transfer from DRT to PRT

In order to transfer subjects from the DRT to the PRT, mice were initially trained over 5 sessions on a continuous reinforcement (CRF) schedule. During this stage subjects were required to make a single nose poke in response to the central stimulus, in order to initiate delivery of the condensed milk reward (22 μl, 10% concentration). These CRF sessions terminated after collection of 100 rewards or if 30 minutes had elapsed.
4.2.5.3 Progressive ratio schedule

After the 5 sessions at CRF, subjects were moved to a progressive ratio (PR) schedule of reinforcement. Under this schedule, the nose poke requirement for food delivery was increased with the following progression: 1, 2, 4, 6, 8 (then further incrementing by 2 nose pokes/ratio), with two trials at each ratio. The progression continued until the subject failed to complete the ratio requirements, determined by the failure to make a further nose poke within 4 minutes of the previous response (timed out). Sessions could also be terminated if 100 rewards had been delivered or if 30 minutes had elapsed, as per the CRF sessions. Subjects carried out three sessions of the PR schedule, across three consecutive days, with performance averaged across the 3 sessions. After the PR schedule subjects were returned to the CRF schedule for 3 days; performance here was averaged over the 3 days. The principal measure of the PRT was the amount of rewards earned, and the ‘break point’ (the maximum ratio a subject reaches during the PRT). Additional measures also included the session duration, the latency to nose poke, and the intra-trial trial NP latency.

4.2.6 Data analysis and statistics

All statistical analyses were carried out using SPSS (version 18.0 for windows). The preference for the condensed milk reinforcer was calculated, based on the volume of condensed milk consumed as a percentage of total liquid consumed, for each of the 5 days of the CMT. During the single nose poke training stage, the principle measure was the number of trials initiated (successfully making a central nose poke when central stimulus is presented). During the double nose poke training stage, the principle measure was the percentage of correct responses (correctly nose-poking the lit stimulus – left or right, after making the initial central nose poke). The principle measure of the delay discounting task proper was the bias to the large reward, defined as the number of times the large reward was chosen as a percentage of the total number of choices within a given delay block. This was taken as a sum of three stable sessions at the stage ‘D’ (the last 3 sessions). Additional measures included; non-started trials (no centre nose poke, so a trial was not started), omissions (centre nose poke, but no choice made), initiation latency (time taken to centre nose poke once the centre light was illuminated), and choice latency (time taken to make a choice nose poke after the centre nose poke), as well as the total number of nose pokes and
panel pushes made throughout the duration of a session. For the progressive ratio task the main measures of interest were the number of rewards collected, the breakpoint of nose poking (the maximum number of nose pokes a mouse would perform for a single food delivery), the overall session duration, the average latency to make the first nose poke for each reward, the latency to collect a reward and the intra-nose poke duration. Behavioural data are presented as mean values (M) along with the standard error of the mean (SEM), unless stated otherwise. All data were analysed by either ANOVA or t tests. All significance tests were performed at alpha level of 0.05 and where significant interactions were identified in the main ANOVA, post-hoc tests using appropriate pair-wise comparisons were performed. For repeated-measures analyses, Mauchly’s test of sphericity of the covariance matrix was applied. Huynh-Feldt corrections were applied as necessary, and adjusted degrees of freedom are provided.

4.3 Results

4.3.1 Milk preference testing

All animals showed an initial aversion to the novel foodstuff (preference of less than 50%), but with subsequent exposures acquired a preference for the milk reinforcer over water (preference of approximately 75%) (Figure 4.3a; main effect of DAY, F_{4,88}=34.91, p<0.001). However, there was no difference between Grb10^{+/p} and WT mice in either their overall preference (Figure 4.3a; main effect of GENOTYPE, F_{4,88}=1.69, p=0.21) nor in the rate at which their preference was acquired (interaction between GENOTYPE and DAY, F_{4,88}=0.69, p=0.42). This was also reflected in the total volume of milk consumed, which increased during the testing procedure (Figure 4.3b; main effect of DAY, F_{4,88}=33.28, p<0.001), but again did not differ between Grb10^{+/p} and WT mice (main effect of GENOTYPE, F_{1,22}=0.34, p=0.57).
Chapter 4 | Assessment of delay discounting behaviour in $Grb10^{+/p}$ mice

**Figure 4.3 Consumption and preference for food reward in $Grb10^{+/p}$ and WT control mice.** Preference for the reward used in the delay discounting task was measured in a simple two-choice consumption test. All animals showed neophobia with an initial preference of less than 50%. However, over the course of testing preference increased until greater than 75% preference for the reward was reached on average for all mice, with no significant difference between $Grb10^{+/p}$ and WT mice (a). This pattern of effects was also reflected in the total volume of milk consumed, which increased during the testing procedure, but also did not differ between $Grb10^{+/p}$ and WT mice (b). Data are the mean ± SEM.

***$P<0.005$. 

### 4.3.2 Delayed reinforcement task

#### 4.3.2.1 Task Acquisition

During the training stages of the DRT (learning to single and double nose poke) the $Grb10^{+/p}$ animals acquired the task at the same rate as WT mice. Subjects took 9 sessions to acquire single nose-poking (successfully initiating >40 trials), and 4 sessions to acquire double nose-poking (successfully completing approximately 70 trials). All animals showed a similar progression throughout the single nose poke training stage, whereby the number of trials initiated (successfully making a centre nose poke when central stimulus was illuminated), improved with each session (Figure 4.4a; main effect of SESSION, $F_{8,176}=14.29$, $p<0.001$), but did not differ between $Grb10^{+/p}$ and WT mice (Figure 4.4a; main effect of GENOTYPE, $F_{1,22}=0.88$, $p=0.36$). During the double nose poke training stage, $Grb10^{+/p}$ and WT mice showed consistently high levels of correct responding to the second stimulus (left or
right) after making the initial central nose poke, which increased with session (Figure 4.4b; main effect of SESSION, $F_{2,42}=23.38, p<0.05$). Both $Grb10^{+/p}$ and WT mice showed a similar proportion of correct responding to the second stimulus (Figure 4.4b; main effect of GENOTYPE, $F_{1,22}=0.05, p=0.83$).

Figure 4.4 Acquisition of the DRT during the single nose poke and double nose poke training stages in $Grb10^{+/p}$ and WT control mice. $Grb10^{+/p}$ and WT mice showed no differences in the number of trials they initiated (successfully making a centre nose poke when the central stimulus was illuminated), during the single nose poke training stage (a). Similarly, $Grb10^{+/p}$ and WT mice also showed very similar and consistently high correct responding to the second stimulus (left or right), after making an initial centre nose poke, during the double nose poke training stage (b). Data are the mean ± SEM.

* $P<0.05$, *** $P<0.005$.

4.3.2.2 Delayed reinforcement behaviour

All subjects successfully learnt the task up to baseline performance. Initially choice for the large reward remained the same when only a small delay was associated with the large reward, during Stage B (Figure 4.5b; main effect of DELAY $F_{2,42}=0.28, p=0.75$). However it was shown that increasing the delay time to the larger reward decreased the likelihood of choosing that reward, at both Stage C (Figure 4.5c; main effect of DELAY, $F_{2,38}=3.73, p<0.05$) and Stage D (Figure 4.5d; main effect of DELAY, $F_{2,38}=10.23, p<0.001$). However $Grb10^{+/p}$ mice were significantly slower to switch their choice to a less delayed, smaller reward than WT mice, during both Stage C (Figure 4.5c; main effect of GENOTYPE, $F_{1,19}=7.78, p<0.05$) and
Stage D (Figure 4.5d; main effect of GENOTYPE, F_{1,19}=7.87, p<0.05). Importantly, when the delay associated with the larger and smaller reward was equal (1s) across all three blocks, all subjects demonstrated a high preference for the large reward (Figure 4.5a; main effect of GENOTYPE, F_{1,21}=0.87, p=0.36), which was maintained throughout the session (main effect of DELAY, F_{2,42}=1.73, p=0.20). Bias scores were excluded from analysis if they had not successfully completed any choice trials, i.e. 100% of choice trials were either non-started trials or omissions, exclusions at each stage were as follows: Stage C n=2 (1 WT, 1 Grb10^{+/p}), Stage D n=1 (1 WT).

**Figure 4.5 DRT performance in Grb10^{+/p} and WT control mice.** Under conditions in which the delay associated with the large and small reward was equal (1s) throughout the session, there were no differences in choice bias between Grb10^{+/p} and WT mice (a). Behaviour of Grb10^{+/p} and WT mice changed with increasing delay,
such that choice bias initially stayed the same when there was only a small difference between the delays for the small and large reward, stage B (b), however choice bias moved away from the response leading to the large reward towards the smaller reward with increasing delay during stage C (c) (where WT, N=10, and Grb10<sup>+/p</sup> N=12 due to exclusions) and stage D (d) (where WT N=10 and Grb10<sup>+/p</sup> N=13 due to exclusions detailed in Section 4.3.2.2). However, there were systematic differences between the groups in their behaviour, such that Grb10<sup>+/p</sup> mice switched their choice to the smaller, less delayed reward, significantly less quickly than controls (c and d). Data are the mean ± SEM of three consecutive stable sessions.

*P<0.05, **P<0.005.

At the baseline (Stage D) of the DRT, Grb10<sup>+/p</sup> and WT mice maintained high levels of responding, as shown by the low levels of non-started and omitted trials (Figure 4.6a & b). All subjects showed an increase in non-started trials as the session progressed (Figure 4.6a; main effect of DELAY, F<sub>2,42</sub>=23.38, p<0.001) with no difference between Grb10<sup>+/p</sup> and WT littermate mice (main effect of GENOTYPE, F<sub>1,21</sub>=0.40, p=0.54). Furthermore the rate of omitted responses did not vary between Grb10<sup>+/p</sup> and WT mice (Figure 4.6b; main effect of GENOTYPE, F<sub>1,21</sub>=2.00, p=0.29).

Grb10<sup>+/p</sup> and WT mice showed an increase in both start (Figure 4.6c; main effect of DELAY F<sub>2,42</sub>=41.01 p<0.001) and choice latency (Figure 4.6d; main effect of DELAY F<sub>2,42</sub>=6.14 p=0.05) as the session progressed. However, all animals showed a high degree of stimulus control and there was no difference between Grb10<sup>+/p</sup> and WT mice on either start latency (Figure 4.6c; main effect of GENOTYPE, F<sub>1,21</sub>=1.44, p=0.24) or choice latency (Figure 4.6d; main effect of GENOTYPE, F<sub>1,21</sub>=0.54, p=0.47). Other behavioral indices were also similar between Grb10<sup>+/p</sup> and WT littermate mice, such as total number of nose pokes (Figure 4.6e; main effect of GENOTYPE, F<sub>1,21</sub>=0.10, p=0.76) and panel-pushes (Figure 4.6f; main effect of GENOTYPE, F<sub>1,21</sub>=2.13, p=0.16, respectively). Furthermore during the ‘forced’ trials (in which no choice was available), both Grb10<sup>+/p</sup> and WT mice made equal responses to the large and small reward-related stimuli at all delays (interaction GENOTYPE x DELAY x CHOICE, F<sub>2,42</sub>=0.35, p=0.65; data not shown). Taken together, these data show that Grb10<sup>+/p</sup> and WT mice had an equivalent ability to learn and perform the DRT. However Grb10<sup>+/p</sup> have altered delay-discounting behaviour, specifically demonstrated by a significant preference for the large delayed reward, which is indicative of decreased choice impulsivity or increased self-control.
Figure 4.6 Ancillary data in the DRT for Grb10\textsuperscript{+/p} and WT control mice. Although many measures were systematically altered by the increasing delay for the large reward, this did not result in significant differences in behaviour between Grb10\textsuperscript{+/p} and WT littermate mice in terms of the number of non-started trials (a) or omitted trials (b) (both out of a possible 8 trials), or the start (c) or choice (d) latency measures, or the number of nose pokes (e) and panel pushes (f). Data are the mean of three baseline sessions ± SEM.

***P<0.005.
4.3.3 Progressive ratio task

4.3.3.1 Task acquisition

Of the 22 mice who took part in the progressive ratio task, 21 subjects successfully completed the task, 1 (WT) subject was excluded for failing to meet the baseline criteria. Mice were initially trained to respond on a CRF schedule for 5 sessions. During the CRF sessions subjects were able to carry out a maximum of 100 trials, which is equal to 100 x 22 µl rewards within each 30 minute test session. Analysis of initial CRF stage revealed that both genotypes achieved the required level of performance per session (see methods for criteria), showing no effect of genotype ($t_{1,19}=0.80, p=0.44$; data not shown).

4.3.3.2 Progressive ratio schedule

Following CRF training subjects were switched to the progressive ratio (PR) schedule, where the number of nose pokes required to initiate a 22 µl reward delivery was gradually increased throughout the session. Subjects performed 3 PR sessions and values from these sessions were averaged together. To demonstrate the effects of the imposition of the PR schedule, performance during the PR sessions were compared to the average of the 3 CRF sessions following PR testing. Imposition of the PR schedule led to a significant reduction in the number of rewards earned relative to CRF responding (Fig. 4.7a, main effect of SESSION, $F_{1,19}=212.15, p<0.001$), and there was no difference between $Grb10^{+/p}$ and WT mice (main effect of GENOTYPE, $F_{1,19}=0.84, p=0.37$). There was also no significant genotype difference in the PR breakpoint, the number of nose pokes required to initiate a reward delivery, (Fig. 4.7b; $t_{19}= 1.42, p=0.17$), suggesting equivalent levels of motivation to work for the reward between the $Grb10^{+/p}$ and WT littermate mice. The decrease in rewards earned in PR sessions relative to CRF sessions was not due to mice running out of time to collect all the available rewards, as the average PR session did not run for the full 30 minutes. There were no significant differences in session duration between the PR and CRF sessions (Fig. 4.7c; main effect of SESSION $F_{3,57}=2.51, p=0.10$) which did not differ between $Grb10^{+/p}$ and WT mice (main effect of GENOTYPE $F_{1,19}=0.18, p=0.68$).
**Figure 4.7 PRT performance in Grb10^+/p and WT control mice.** Imposition of the PR schedule led to a significant reduction in the number of rewards earned, in comparison to CRF responding, for both WT and Grb10^+/p mice (a). Grb10^+/p and WT mice showed equivalent break points, the maximum number of nose pokes.
they were willing to make to earn a reward, during the PR schedule (b). This PR was not due to mice running out of time, as there were no significant differences in session duration between the PR and CRF sessions (c). Results also demonstrated that Grb10^{+/p} and WT littermate mice showed equivalent levels of stimulus control in the PRT evidenced by no differences in the latency to complete the first nose poke (d), the intra-trial nose poke latency (e) or the latency to collect the reward (f). Data are the mean of three baseline sessions ± SEM.

***p<0.005.

Analysis of additional measures of the PRT during the PR schedule revealed that the Grb10^{+/p} and WT littermate mice also had comparable levels of stimulus control. Specifically, subjects showed equivalent latencies to make the first nose poke (Fig. 4.7d; main effect of GENOTYPE, t_{1.19}=-1.08, p=0.29), equivalent intra-trial nose poke latencies (Fig. 4.7e; main effect of GENOTYPE t_{1.19}=-1.37, p=0.18) and equivalent latency to collect the reward (Fig. 4.7f; main effect of GENOTYPE t_{1.19}=0.34, p=0.74). These data therefore demonstrate that Grb10^{+/p} and WT mice have an equivalent motivation to work for the reward used in the DRT.

4.4 Discussion

The principal aim of this chapter was to assess impulsive choice behaviour in Grb10^{+/p} mice, which was achieved using a delay-discounting paradigm. This behaviour was investigated as the overlapping expression patterns in brain regions related to impulsivity between Grb10 and Nesp, the reciprocity in imprinting status and the fact that previous research had showed that Nesp^{m/+} mice to have altered behaviour in the DRT (Isles et al, unpublished results), suggests that Grb10 may influence the same behaviours as Nesp. For consistency with the previous findings, the design of the DRT was the same, and based on a block design where the delay associated with the large reward increased between blocks, but was consistent within a block. This design encouraged flexible ‘trial discrete’ choices, which minimises choices being based on rigid habitual behaviour (Evenden and Ryan, 1996). Using this operant method, a cohort of Grb10^{+/p} and their WT littermate mice were used to demonstrate that Grb10^{+/p} subjects had altered delay-discounting behaviour, specifically an increased tolerance of longer delays before reward was delivered which can be interpreted as decreased choice impulsivity or increased self-control. However, before pursuing this
argument, there are other possibilities that could account for the difference in behaviour; issues such as general competence in performing the task, motivation for the reward or different levels of satiation between \( \text{Grb10}^{+/p} \) and their WT littermate mice.

The patterns of behaviour shown in the DRT were not due to differences in competence at performing the task. \( \text{Grb10}^{+/p} \) animals acquired the task at the same rate as WT littermate mice, and demonstrated an equally high degree of stimulus control as indicated by latency measures. Additionally, the difference in DRT performance between \( \text{Grb10}^{+/p} \) and WT mice was not related to differences in experiencing the information trial contingencies. In the ‘forced’ information trials (in which no choice was available), both \( \text{Grb10}^{+/p} \) and WT mice made equal responses to the large and small reward-related stimuli at all delays. This indicated that subjects received the same information in order to accurately complete the task. Both WT and \( \text{Grb10}^{+/p} \) subjects showed equivalent levels of stimulus control and showed no deficit in the ability to perform both the DRT and the PRT, demonstrated by comparable latencies to make choices in the DRT, collect the reward and make the initial nose poke, and for the intra-trial nose poke latency in the PRT. Taken together, these measures suggested that the only factor controlling the difference in behaviour between \( \text{Grb10}^{+/p} \) and WT mice on the DRT, was the contingency between delay and reward and the resultant choice made by the \( \text{Grb10}^{+/p} \) subjects.

When using food reinforcement in cognitive tasks with mice, it is important to establish if mice demonstrate the equivalent levels of motivation for the reward. This is especially important in the DRT for two reasons. Firstly, where the perceived value of the reward is paramount to the task, altered motivation for the reward could generate similar patterns of results to those found here. Secondly, the main effect found in relation to decreased choice impulsivity was most profound in the last block of a session (where the delay was greatest), and altered motivation would also be decreased due to satiety effects. Therefore reward motivation was specifically assessed using a progressive ratio task (PRT), which measured how much effort a subject will make to earn a reward, in the paradigm used here the amount of nose pokes needed to receive a single reward was increased throughout a session. Results demonstrated that both WT and \( \text{Grb10}^{+/p} \) subjects successfully performed the task; under continuous reinforcement conditions (where a reward was earned after a single nose poke) subjects earned a high number of rewards. However under
the PR schedule, all subjects achieved significantly fewer rewards, with Grb10+/p and their littermate mice showing equivalent ‘breakpoints’, the measure of motivation to earn rewards. Therefore motivation for reward, as indexed by the PRT, was equivalent between Grb10+/p and WT mice, and also would not account for the pattern of performance found in the DRT.

Evidence from both the DRT and PRT would suggest that satiation also did not contribute to the behavioural differences in the DRT observed between Grb10+/p and WT littermate mice. On average in the DRT, subjects were able to consume a maximum of 1.62 ml (based on choosing the large reward in all 24 choice trials, in addition to 12 forced trials), an amount much less than the amount of reward consumed in the CRF stage of the PRT. Here, both WT and Grb10+/p subjects consumed approximately 80 rewards, equivalent to 1.76ml (80 x 22 μl) of reward, significantly more than the maximum possible in the DRT. Thus, in a situation where there was no contingency on reward delivery (either by increased delays in the DRT or ratio increases in the PRT), Grb10+/p and WT littermate mice consumed equal amounts of reward. Therefore, the decreased choice impulsivity shown by Grb10+/p mice in the DRT was not due to differences in motivation or satiation for the reward, but most likely due to increased tolerance of the reward-delay underlying altered impulsive behaviour.

Discounting paradigms have been used extensively to characterise decision making in a variety of animal species. Tests of delay-discounting are associated with the measurement of cognitive impulsivity or ‘impulsive choice’ (Ho et al., 1999, Winstanley et al., 2003), whereby the selection of a smaller immediate reward in preference to larger delayed reward has been considered to reflect ‘impulsive’ choice, whereas the opposite bias toward delayed gratification has been taken to indicate increasing ‘self-control’ (Rachlin et al., 1991). Based on this premise, the results presented in this chapter suggest that Grb10+/p mice are less impulsive relative to their littermate controls (showing a preference for delayed gratification), in contrast to previous research showing that Nesp<sup>m+/</sup> mice are more impulsive relative to their littermate controls (showing a preference for immediate gratification)(Isles et al, unpublished results). This therefore demonstrates a reciprocal effect on this type of behaviour with maternally expressed Nesp promoting self-control, and paternally expressed Grb10 promoting impulsivity.
Whilst using delay-discounting as a measure of impulsive choice has proved useful in clinical research with humans, it is also important to consider risk-sensitivity of animals (Kalenscher, 2007). Whilst risk-taking and impulsive behavior are often thought as one in the same (Xu et al., 2013) and indeed are usually correlated (Simon et al., 2009), in delay discounting tasks they may dissociate. It is thought that animals may equate temporal distance with collection risk, therefore a temporally proximal reward may be preferred over a temporally distant reward, in the same way as a certain reward is preferred over a less certain reward, since delayed benefits may be lost during waiting time and are hence realised with less confidence (Kalenscher, 2007). Ethobiologically, animals tend not to chance waiting for food if there is the option of an immediate food source available; due to the potential for predation or competition that may result in danger or loss of the food (Green and Myerson, 1996, Kacelnik and Bateson, 1996). Therefore it has been suggested that the discounting of delayed reward shown in animals is due to interpreting the large, delayed reward as a more risky outcome (Hayden and Platt, 2007, Kalenscher, 2007).

As a result of these theoretical and empirical considerations, it has been suggested that choosing the small immediate reward would be a ‘safer’ option, whereas choosing the larger reward with a degree of uncertainty (delay) would be a ‘risky’ option (Kalenscher, 2007). Consistent with this hypothesis, increasing the delay time to the larger reward decreased the likelihood of choosing that reward for both Grb10+/p and WT littermate mice. However, Grb10+/p and WT mice also showed significant differences in the extent to which they switched their preference to the smaller, less delayed reward in response to increased delay to the larger reward. Based on the risk-taking interpretation of delay-discounting behaviour, it could therefore be concluded that Grb10+/p mice showed significantly increased risk-taking behaviour in the DRT. Importantly, when the ‘risk’ element was removed in the equal delay manipulation, where the delay associated with the larger and smaller reward was equal (1s) across all three blocks, all subjects demonstrated an equal preference for the larger reward throughout the session. Under these conditions, the choice became much simpler, and demonstrated that the difference in behaviour between Grb10+/p and WT mice was only apparent when there is a trade-off between the benefit of the larger reward and the risk associated with a long delay.
Although the data presented in the current chapter showed a significant effect of genotype in the delay discounting task, it is somewhat surprising that a GENOTYPE x DELAY interaction was not found. If the behavior of the Grb10^{+/p} mice was a function of the delay one would expect a significant interaction to be found; the lack of interaction is likely to be an effect of a potential deficit of the WT mice at the no delay (1s) stage. Whereby the WT group show an unexpectedly low preference for the large reward at the 1s stage (Figure 4.5b and 4.5c), where preference is approximately 70-80%, previous research reports 90-100% preference for the large reward when there is no delay (Evenden and Ryan, 1996). However it is important to note that this effect is driven by the behaviour of a small number of subjects (1-3) and is not seen across the whole cohort. Furthermore the initially low levels of responding at the 1s stage may be a result of a carry-over effect; whereby subjects may initially still associate the large reward with a long delay based on the end of the last session.

The data presented here from the DRT, along with the previous Nesp^{m/+} data (Isles et al, unpublished results), represent the first demonstration of imprinted genes influencing delay discounting behaviour. Moreover the behaviour demonstrated by Grb10^{+/p} mice is opposite to that demonstrated by Nesp^{m/+} mice; whereby Nesp^{m/+} mice were significantly quicker to switch their choice to the small immediate reward (Figure 4.1). This directionality of empirical effects is concordant with the theoretical hypotheses, such that maternally expressed imprinted genes are thought to promote the tolerance of riskier outcomes, whereas paternally expressed imprinted genes are thought to reduce the tolerance of riskier outcomes (Wilkins and Haig, 2003). Moreover, the patterns of behaviour also support the assertion that risk-taking behaviour is a substrate for the evolution of genomic imprinting independent from the more familiar substrate of resource allocation between mother and offspring (Constancia et al., 2004), as these models are free from confounding gross effects on in utero or pre-weaning growth (Plagge et al., 2005, Garfield et al., 2011), supporting the rationale for the confliction hypothesis of genomic imprinting (Constancia et al., 2004).

Expression of Nesp and Grb10 in the locus coeruleus and dorsal raphe nucleus (Plagge et al., 2005, Garfield et al., 2011) suggest that these genes may exert their influence on delay-discounting behaviour via the noradrenergic and/or serotonergic system. Moreover, expression of Grb10 in the ventral tegmental area and substantia nigra pars
compacta may also imply that this gene exerts influence on delay discounting behaviour via
the dopaminergic system (Garfield et al., 2011). Each of these monoamine
neurotransmitters systems have been shown to be important in the modulation of
behaviour in discounting paradigms (Cardinal 2006, Robinson et al., 2008), as they are key to
the control of choice impulsivity. Whereby evidence from animal studies indicate that
alterations to the serotonergic, dopaminergic and noradrenergic systems causes altered
delay discounting behaviour (Cardinal 2006). Furthermore the areas of the mid-brain that
Nesp and Grb10 are expressed in have been identified as ‘hot-spots’ of imprinted gene
expression in a recent genome-wide screen for parental-biased allele expression in the
rodent brain (Gregg et al., 2010), therefore suggesting that other imprinted genes expressed
in these areas, may also influence discounting or impulsive behaviour.

The data presented here, show that Grb10+/p mice have altered delay-discounting
behaviour, showing a significant preference for larger, more delayed rewards. This is
opposite to that found in the previously tested Nesp^m/+ mice, whom showed a significant
preference for the small immediate reward. Based on the different interpretations of delay-
discounting tasks, this behaviour could be interpreted as the genes having conflicting choice
impulsivity or risk-taking behaviour. Further testing in assays of both impulsivity and risk-
taking are required to determine if the altered behaviour observed in Nesp^m/+ and Grb10+/p
mice is a facet of impulsivity or risk-taking behavior (see Chapter 6). Nevertheless, the data
shown here suggests that these behaviours may be a substrate for imprinted gene action;
specifically paternally expressed Grb10 compared with maternally expressed Nesp (Isles et
al, unpublished results). Therefore, these data provide a novel, and predicted mode of
action for genomic imprinting in the brain, that is dissociated from resource allocation
between mother and offspring.
4.4.1 Summary of key results from Chapter 4

- *Grb10*+/− and WT littermate mice were able to learn and accurately complete the delayed reinforcement and progressive ratio tasks.

- *Grb10*+/− mice had altered delay-discounting behaviour, showing a significantly higher preference for the large, delayed reward, in a delayed-reinforcement task; which is opposite to that found previously in *Nesp*<sup>m/+</sup> mice.

- The behaviour found in the delay-discounting task was not a result of differing motivation for the reward, as *Grb10*+/− and WT mice showed equivalent motivation to earn rewards in the progressive ratio task.
Chapter 5 – Development of a novel test of risk-taking: the Predator Odour Risk-Taking (PORT) task

5.1 Introduction

Many paradigms used to assay risk-taking behaviour in humans have been successfully translated into tasks for use with rats and mice, for example the balloon-analogue risk test (BART; Ashenhurst et al., 2012), the Iowa gambling task (de Visser et al., 2011) and the probabilistic-discounting task (Onge et al., 2012). Moreover, results of a delay discounting task, which produced dissociable behaviour between Nesp$^{m/+}$ (Isles, et al. unpublished results) and Grb10$^{m/+}$ mice (See Chapter 4), can also be interpreted as demonstrating risk-taking behaviour. Most current rodent tests of risk-taking have successfully translated human risk-taking tasks where loss or attenuation of a reward is the potential ‘risk’. However, whilst these paradigms are useful for examining decision-making generally, many (Simon et al., 2009, Choi and Kim, 2010, Franks et al., 2012) have argued that these tasks do not examine real-life risk-taking behaviour, where there is a trade-off between a benefit (or reward) and a separate cost (or risk). Thus, the current range of rodent risk-taking tasks may not capture a ‘real-world’ risky situation relevant to an animal.

The importance of ‘real-world’ animal decision-making tasks is being increasingly recognised in the pursuit of accurately measuring risk-taking in laboratory animals (Simon et al., 2009, Choi and Kim, 2010). This problem has recently begun to be addressed by the development of behavioural tasks that assess the degree to which a separate factor, such as probability of a foot-shock (Simon et al., 2009) or safety of a dark environment (Franks et al., 2012) is traded against gaining a food reward. The current chapter describes the development of a novel test of risk-taking that exploits the conflict between two of the most biologically relevant aspects of a rodent’s life: the motivation to obtain food vs. the risk of meeting a predator. Thus, the novel test of risk for mice, the predator odour risk-taking (PORT) task, measures the trade-off between two separate factors; an unpleasant or unwelcome occurrence (potential danger), and something rewarding (obtaining food); therefore taxing a more ethobiologically valid type of risk-taking. In the PORT task water-
restricted mice were trained to traverse a 3 chambered apparatus to collect a 10% condensed milk reward, but were required to pass through a compartment which contained standard bedding material that was mixed with an odour from a major mouse predator: rat, cat or fox. Mice have an innate fear of the odour of their major predator species and show an escape response in their presence (Belzung and Griebel, 2001, Kavaliers et al., 2001, Fendt and Endres, 2008). For example, the synthetic odour from fox anal secretions, 2,4,5-trimethylthiazoline (TMT), has been shown to induce defensive behaviours including avoidance and escape, increased acoustic startle responding, and elevated blood corticosterone (Blanchard et al., 2003, Hebb et al., 2003, Fendt and Endres, 2008, Galliot et al., 2012). These studies have investigated the effects of predator odours in terms of avoidance of the stimuli rather than balancing the tension between their anxiogenic nature and the drive to reach a goal. The ability to appropriately assess such risks (exposure to a predator) and weigh them up against potential rewards (food) is a crucial component of optimal decision-making, a cost-benefit analysis. In the PORT task, the anxiogenic nature of these odours (the cost) was utilised in a conflict situation whereby the mice were motivated to seek a food reward (the benefit).

In defining and validating the PORT task a mouse strain of mixed genetic background, $F_2$(C57BL/6J*CBA/Ca) was used, as this is the strain on which both Nesp$^{m/+}$ and Grb10$^{m/+}$ mice are now maintained. As well as the characterisation the PORT task itself, this chapter will also address a number of ancillary measurements, carried out in order to ensure that the PORT task accurately measured risk-taking behaviour. These included an assessment of locomotor activity, an assessment of fear/anxiety using the elevated-plus maze (EPM) and an assessment of potentiated startle using exposure to a predator odour (TMT).
5.2 Methods

5.2.1 Subjects

13 male $F_2$[C57BL/6J*CBA/Ca] mice were used, produced from in-house breeding of $F_1$[C57BL/6J*CBA/Ca] with $F_1$[C57BL/6J*CBA/Ca]. Mice were 6 months old at the start of testing (mean weight: $30.38 \pm 0.49$ g). Animals had ad libitum access to standard lab chow, but water access was restricted to 2 hours during testing (given after testing). Details of general housing, husbandry and handling leading up to the experimental period were as described in Section 2.3.

5.2.2 PORT Apparatus

The apparatus used in the PORT task is shown and briefly described in Chapter 2, Section 2.4.6. The apparatus consisted of a white Perspex arena divided equally into three chambers, with each chamber measuring $30 \times 30 \times 40$ cm (depth x width x height), arranged in a row such that the middle chamber could be entered from either of the end chambers (Fig. 5.1). Guillotine doors, operated with a pulley system, controlled access to the middle chamber (doorway was $5 \times 5$ cm, width x height). The central chamber was where different odours were introduced; whilst the outer (left and right) chambers were used as either the start chamber or the reward chamber (counterbalanced between subjects). For example, a mouse starting from the left hand chamber (designated the ‘start chamber’) would cross the middle chamber and collect the reward in the right hand chamber (designated the ‘reward chamber’). Reward was a $100 \mu l$ aliquot of 10% solution of condensed milk (Nestlé, U.K.) placed in a small pot attached in the centre of the reward chamber floor by Velcro. 10% condensed milk has been shown previously to be extremely motivating for mice (Humby et al., 1999, Isles et al., 2003). Predator odours (see below) were mixed with an equal amount of regular bedding material (wood shavings) and distributed evenly over the entire floor of the middle chamber prior to a trial.

Data were collected using EthoVision Observer software (Version 3.0.15, Noldus Information Technology, Netherlands), as described in Section 2.4.6, with a specific virtual arena and zone configurations. Thus, each chamber was designated as a zone (left, middle, right), and a circular area around the reward container was also specified as an independent
zone (5cm diameter). Calculations based on the start direction (left or right) were used to determine which data would be identified as collected in the start or rewarded chamber, and reward collection. The EthoVision tracking system performed calculations over a series of frames (12 frames/sec) to derive a set of quantitative descriptors about the movement and location of subjects, and variables such as duration, movement, entries and latency to first enter each zone of the chamber were determined by the EthoVision programme in terms of location of the greater body-proportion of subjects. Tracking of the subject was calibrated for the test apparatus prior to testing using non-experimental mice of the same body size and coat colour as the experimental subjects. Data were analysed as total values for each test trial and in 5-minute intervals for the habituation session. From the range of parameters generated, the primary measures analysed were the latency to collect the reward from the start of a trial, the duration of time spent in each chamber, the distance and movement velocity, and the amount of rearing in each chamber was also measured. Rearing behaviour was automatically determined by the EthoVision system by registering a decrease in the surface area of the object being tracked. To confirm the accuracy of the digitised tracking system, all sessions were also recorded using DVD HD recorders (Sony Corp, U.K.) for further analysis if required.

5.2.3 Predator odours

For each test trial an equal amount (500 ml) of odour/bedding mixture was placed into the middle chamber prior to the test starting. For the mouse and rat odours, this consisted of bedding material (wood shavings) collected from animal cages, whereas for the cat and fox odour mixtures, clean standard bedding material was mixed with the odorising substance/material. For each odour/bedding mixture the volumes of bedding, amount of odorising material and the time between mixing and using was standardised. This was especially relevant for the rat and cat odours which were collected from the cages in other vivaria. All odour mixtures were made 2 days before use and were kept in sealed bags in a room separate to the holding or test rooms, and only the amount required was collected before a trial.
Control bedding. 500 ml of clean standard mouse bedding material.

Mouse (self) odour. 500 ml of the bedding material from the subjects’ own home cage was collected on the day of testing and the same bedding was used for all subjects housed in the same home cage.

Rat odour. Bedding material was collected from the cages of male Lister-hooded rats (housed in pairs); 1 week old on day of collection.

Cat odour. A pigeon liner was placed in a cat enclosure, housing two male cats, for 3 days. Approximately 100 ml of fur was also collected when the cats were being groomed. The liner was cut into small squares and, with the fur, mixed with the standard bedding material and left in a sealed bag for 3 days. Cat odour has previously been shown to be aversive to mice (Kavaliers et al., 2001).

Fox odour. 2,4,5-trimethylthiazoline (TMT) (Contech Inc, Canada), a synthetic predator cue isolated from red fox (Vulpes vulpes) anal secretions, was used. A 10% TMT solution was made by diluting TMT in water and Tween (30 μl). 500 μl of the solution (in 50 μl aliquots) was pipetted on to 10 pieces of blotting paper (5 cm²), which were then placed in a bag containing 20 L of wood shavings. Therefore, each 500 ml aliquot of standard bedding material placed into the middle chamber of the apparatus (volume of 3600 cm³) was equivalent to 12.5 μl of 10% TMT/cm³, which is consistent with the concentration levels of TMT used previously to engender aversion and anxiety (Galliot et al., 2012).

5.2.4 Procedure

Initial training took place over an 8 day period consisting of 6 days habituation to the reward to be used (10% condensed milk), habituation to the test environment and finally acquisition of the basic test procedure. All experiments were conducted in daily sessions, home cage water was always given once testing was completed. On each day of training and testing, subjects were moved from the vivarium and kept in a separate room adjacent to the testing room until required. Both the holding and test rooms were illuminated by dim lights and care was taken to minimise physical disturbance or excessive noise. Before and between trials, mice were kept in a cage in the test room adjacent to the apparatus. Between individual mice and between trials the apparatus was cleaned thoroughly with a
1% acetic acid solution, and odour/wood shaving mixtures were removed by vacuum cleaner.

5.2.4.1 Habituation to the reward

Animals were habituated to the condensed milk reward using the milk preference test protocol as outlined in Section 2.3.5.

5.2.4.2 Habituation to the test apparatus

Subjects were placed in the centre of the middle compartment of the apparatus, with both the doors open, and allowed to explore the 3 chambers for a period of 20 minutes. In order to keep the habituation trials consistent with the acquisition and test trials, the chamber was cleaned with 1% acetic acid and 500 ml of clean standard mouse bedding (wood shavings) was placed in the middle chamber before each mouse. There was no reward present during these habituation sessions. The time spent and the numbers of entries into each chamber (left, middle and right) were determined for each 5 minute period of the habituation session.

5.2.4.3 Acquisition

In this stage of training subjects learnt that reward (100 μl of 10% condensed milk) was available within the test apparatus. Each subject completed 5 consecutive trials, whereby they were placed in one of the outer chambers (‘start’), the doors were opened and the subject was free to explore the 3 chambers and collect the reward situated in the opposite (‘reward’) chamber. Direction of travel was counter-balanced between mice, such that some subjects moved from left to right and others, right to left, but direction was fixed for each individual subject throughout testing. There was no time limit on these trials and each trial was stopped manually when the subject was observed to have collected the reward. The subject was then removed and placed in a holding box before the reward was replenished and the apparatus cleaned using 1% acetic acid. In order to keep the acquisition trials consistent with the probe trials, 500 ml of clean standard mouse bedding was placed in the middle chamber before each trial. The latency to reach the reward chamber and collect the reward from the start of each trial the duration of time spent in
each chamber, the distance moved, movement velocity, and the amount of rearing in each chamber were determined for each trial.

5.2.4.4 Predator odour risk testing

Trials were carried out in the same way as the acquisition stage, whereby subjects were placed in the start chamber and were required to cross the middle chamber in order to collect the reward situated in the opposite chamber. Trials ended when the subject had consumed the reward; with a maximum trial duration of 10 minutes. On each day of testing, all subjects completed 6 trials: 3 training trials in the morning with control bedding and 3 test trials in the afternoon with an odour/bedding mixture (minimum delay between each set of trials was 2 hours). Thus, for the 3 consecutive training trials the middle chamber contained 500 ml of clean standard mouse bedding and for the 3 consecutive test trials, 500 ml of ‘predator odour bedding’ or ‘self odour bedding’ was placed in the middle chamber. After each subject had completed either the control or test trials, the bedding was removed, and the chamber cleaned with 1% acetic acid solution. The self odour test session, using mouse bedding, was conducted on the day following acquisition, and subsequent sessions (with rat, cat and fox odours) with 3 day intervals. Further tests to investigate altered motivation were also performed with a 1% condensed milk solution as the reward instead of the normal 10% concentration. On each day of testing the animals received 1% milk in both the 3 training trials in the morning, and the test trials in the afternoon. Test sessions for different predator odours were carried out in separate sessions, 3 days apart, counter-balanced for order. As previously, the main parameters assessed in each trial were the latency to reach the reward chamber and collect the reward from the start of each trial, the duration of time spent in each chamber, the distance moved, movement velocity, and the amount of rearing in each chamber.

5.2.5 Locomotor activity (LMA)

In order to assess reactivity to novelty and the general locomotor activity mice were assessed with a single 120 minute activity session. The LMA was carried out using the apparatus described in Section 2.4.1, and standard procedure outlined in Section 3.2.2.
5.2.6 The elevated-plus maze (EPM)

In order to measure general levels of anxiety, mice were assessed on the EPM, using the apparatus described in 2.4.4. Subjects were placed at the centre of the EPM facing one of the exposed open arms and allowed to explore the maze over a single session of 5 min. To optimize data analysis using the EthoVision observer software, the different parts of the EPM were divided into 5 virtual zones representing the 2 open arms, 2 closed arms and the middle section (8 x 8 cm, length x width) from which all arms originated. Data from the 2 open arms was combined to generate a single open arm value and a similar cumulative closed arm value was also produced. The main parameters used as indices of anxiety-related behaviour were the proportion of time spent exploring the open arms (relative to the time spent on both open and closed arms) and the number of entries onto the open and closed arms. In addition, the following parameters were manually scored: number of stretch attend postures (SAP, defined as an animal keeping its hindquarters in the closed arms but stretching forwards onto an open arm) and number of head dips from the open arms (looking over the edge of an open arm).

5.2.7 Anxiety-induced Acoustic Startle (ASR) and Prepulse Inhibition (PPI).

It has been shown that ASR is increased in the presence of a predator odour, such as TMT, whereas PPI remains unaffected (Hebb et al., 2003). Therefore, mice were assessed using the ASR/PPI chambers as described in Section 2.4.5. Subjects were assessed in 2 separate test sessions, a week apart, immediately following 10 min exposure to either untainted standard bedding material (control condition) or fox odour tainted bedding material, mixed at the same concentration of TMT as used in the PORT task (see above). The order of odour presentation was counter balanced between mice. ASR and PPI were monitored using SR-Lab apparatus (San Diego Instruments, U.S.A), according to previous method (Geyer and Dulawa, 2003). White noise stimuli were presented via a speaker mounted in the roof of a sound-attenuating chamber, 12 cm above the subject. Animals were placed in a Perspex tube (internal diameter 35 mm) mounted onto a Perspex plinth. Startle responses were transduced and digitised by a piezoelectric transducer linked to the computer and placed directly under the centre of the Perspex tube.
A session consisted of a 5 min habituation period followed by 2 blocks of acoustic stimuli presented against a background white noise (65 db, A scale) with the startle amplitude set at 120 db in the first block (13 pulse-alone, 18 prepulse and 4 no stimulus trials) and a pseudorandom distribution of pulse-alone trials of increasing amplitude (3 trials each at 80, 90, 100, 110 and 120 db and 5 no stimulus trials) in the second block. Stimuli were presented in a pseudorandom manner every 10 s. Pulse-alone trials consisted of a 40 ms 120 db startle stimulus and a prepulse trial consisted of an initial 20 ms prepulse stimulus at 4, 8, or 16 db above background and a 30 ms, 120 db startle stimulus, 70 ms after the prepulse offset. In the first block of stimuli, prepulse trials at the different intensities and no-stimulus trials were pseudorandomly distributed between the pulse-alone trials. The whole body startle response to the pulse-alone trials and the gating (i.e. inhibition) of responding due to the presentation of prepulse stimuli was recorded as the average startle over a 65 ms period timed from the onset of the startle pulse, and each trial type was averaged together. PPI was calculated as the percentage reduction in startle amplitude between prepulse trials and pulse-alone trials.

5.2.8 Data analysis and statistics

All statistical analyses were carried out using SPSS 18.0 for Windows. For each of the different test odour sessions (control bedding, self, rat, cat and fox odours), mean values were determined by averaging each of the 3 probe trials together. The following parameters were analysed for each session: latency to enter the reward chamber from the start of the trial, duration within the start, middle and reward chambers, distance moved in the start and middle chambers, the amount of rears in the start and middle chambers and the velocity of movement in the start, middle and reward chambers. Data comparing the effect of different predator odours on behaviour was analysed by ANOVA with repeated measures of ODOUR (self, rat, cat and fox) and CHAMBER (start or middle). To investigate the effect of reducing the reward concentration, parameters were analysed by ANOVA with a repeated measures of ODOUR (control, rat) and CONCENTRATION (10%, 1%). Habituation data were analysed by ANOVA with a repeated measures of TIMEBIN (0-5, 6-10, 11-15, 16-20) and CHAMBER, acquisition data with repeated measures of TRIAL (1-5) and CHAMBER.
and habituation to the reward by ANOVA with factors of DAY (1-6) and solution (water, 10% condensed milk). Beam breaks from the locomotor activity session was analysed by ANOVA with a repeated measure of BIN (0-30, 31-60, 61-90, 91-120 minutes from start of the session) and the elevated plus maze data (entries /arm) by ANOVA with repeated measures of ARM (open, closed) and BIN (1,2,3,4,5 minutes from the start of the session). Data from the duration spent in the arms of the elevated plus maze violated the ‘independence’ assumption for parametric tests; therefore a Wilcoxon signed rank test was used as a nonparametric substitute. Data from the first block of ASR testing were analysed by ANOVA with factors of ODOUR (control or fox) and TRIAL (1-13) for the pulse-alone trials, and TRIALTYPE (4db prepulse, 8db prepulse and 16 db prepulse) for degree of PPI). Data from the second block of stimuli was analysed by ANOVA with factors of ODOUR and AMPLITUDE (70, 80, 90, 100, 110 and 120 db). Pearson’s correlations were used to assess relationships between key parameters from each of the different tests. For repeated-measures analyses, Mauchly’s test of sphericity of the covariance matrix was applied. Huynh-Feldt corrections were applied as necessary, and adjusted degrees of freedom are provided. Post hoc pairwise comparisons were performed using the Bonferroni test, with alpha values of <0.05 regarded as significant.

5.3 Results

5.3.1 Milk preference testing and acquisition of PORT task

Before the F₂[C57BL/6J*CBA/Ca] mice could be assessed for risk-taking behaviour in the PORT task, they were first habituated to the 10% condensed milk reward solution and the test apparatus. Following this, subjects underwent training to acquire the basic procedure of traversing the middle chamber, on exiting the start chamber to collect reward in the furthermost chamber. All mice showed a significant preference for the condensed milk reward solution over water, reaching >75% preference by the end of the habituation test (Fig 5.2a; main effect of SESSION, $F_{4,48}=22.89$, $p<0.001$) and consuming on average 1.4 ml of solution (Fig 5.2b; main effect of SESSION, $F_{4,48}=1.65$, $p<0.001$). During the 20 minute habituation session, mice readily explored the 3-chamber apparatus, making more entries into the middle chamber (Fig 5.2c; main effect of CHAMBER, $F_{2,24}=14.62$, $p<0.001$), as might
be expected as the mice traversed the apparatus, which was also reflected by an increase in the amount of time spent in this chamber (Fig 5.2d; main effect of CHAMBER, $F_{2,24}=97.54$, $p<0.001$). During acquisition of the PORT task procedure, the mice were trained to traverse the apparatus from the start chamber to collect 100 μl of 10% condensed milk in the reward chamber. Results show that mice readily traversed the middle chamber containing clean standard (control) mouse bedding, taking on average <2 minutes to collect the reward across all 5 trials (Fig 5.2e). Latency to collect reward increased with trial (Fig 5.2e; main effect of TRIAL, $F_{4,48}=10.41$, $p<0.005$).

Figure 5.2 Reward habituation and acquisition of the PORT task procedure by male $F_2$[C57BL/6J*CBA/Ca] mice. During habituation to the reward, all mice showed an increasing preference for the 10% condensed milk reward over water (a), consumption of condensed milk increased across testing days (b). During the 20 min habituation session to the 3-chamber apparatus $F_2$[C57BL/6J*CBA/Ca] mice readily explored the different chambers of the PORT task apparatus, entering each compartment a number of times (c), but spending most time in the middle compartment (d). During acquisition, the mice were trained to cross the PORT task apparatus to collect the reward, passing through the middle chamber which now had clean bedding material on the floor. All the mice traversed the chambers quite rapidly across all 5 trials (e). Error bars show mean±SEM. N=13. ***P<0.005.
5.3.2 Predator odour alters behaviour in PORT task

Following acquisition, $F_2$[C57BL/6J*CBA/Ca] mice were assessed for risk-taking behaviour by mixing the bedding material in the middle chamber of the PORT task apparatus with rat, cat or fox odours (Fig. 5.3a). Control sessions were also carried out, with clean untainted bedding, as well as bedding from the subject’s own cages. Mice were given 3 training trials with control bedding in the central chamber in the morning and then 3 test trials with bedding mixed with a control (own bedding) or predator odour in the afternoon. For ease of viewing the 3 trials of each test session have been collapsed to yield single values. In the presence of a predator odour mice were more reluctant to collect the food reward relative to control (own) odour as evidenced by the EthoVision tracks and the overall collection latencies (Fig. 5.3b; main effect of ODOUR, $F_{3,36}=11.93$, $p<0.001$). A breakdown of the overall duration indicated that the predator odour had a similar effect on time spent in the start chamber (Fig. 5.3c; main effect of ODOUR, $F_{3,36}=6.32$, $p<0.001$) but not the middle chamber (Fig. 5.3d; main effect of ODOUR, $F_{3,36}=3.447$, $p=0.06$); suggesting that increased latency was due to fear to leave the start chamber, not increased exploration of the odour. Pairwise comparison of $F_2$[C57BL/6J*CBA/Ca] data revealed that total latency to collect was significantly increased for rat odour ($p=0.020$), fox odour ($p=0.003$) and cat odour ($p=0.009$) compared to control (self) odour (Fig. 3b).
Figure 5.3 Effects of different predator odours on risk-taking behaviour in male $F_2$[C57BL/6J*CB6/Ca] mice. Mice were assessed for the time taken to cross the PORT task apparatus in the presence of different odour/bedding material combinations, including their own given bedding. Mice were given 3 trials during each phase of testing and for ease of viewing we have collapsed the trials of each probe session to yield single values. (a) Example traces for a $F_2$[C57BL/6J*CB6/Ca] mouse to traverse the PORT task apparatus. Overall, mice were slower to collect the reward in the presence of a predator odour (b). Increased latencies to collect the reward were mainly as a result of increased emergence times from the start chamber (c), rather than investigation of the novel odours in the middle chamber (d). Error bars show mean ±SEM. N=13.  

***P<0.005.

5.3.3 Value of food reward influences behaviour on PORT task

The effect of altering the value of the food reward on behaviour of $F_2$[C57BL/6J*CB6/Ca] mice in the PORT task was assessed by reducing the concentration of the condensed milk from 10% to 1%. This test was carried out on both a control and predator odour (rat) background on separate test days. Reducing the concentration for the food reward led to significantly increased reward collection latencies (Fig. 5.4a; main effect of CONCENTRATION, $F_{1,12}$=14.50, p<0.05). However, there was no significant interaction
between altering the reward value and the presence of a predator odour ($F_{1,12}=25.80$, $p=0.91$), suggesting that the effects of reduced reward concentration in the presence of predator odour were not additive. Again this slowing of behaviour was reflected in the component parts of the latency to collect the reward, with animals taking longer to leave the start chamber (Fig. 5.4b; main effect of CONCENTRATION, $F_{1,12}=7.03$, $p=0.02$) and an increased duration in the middle chamber (Fig. 5.4c; main effect of CONCENTRATION, $F_{1,12}=6.15$, $p=0.03$). Pairwise comparison of $F_{2}[C57BL/6J*CBA/Ca]$ data revealed that total latency to collect was significantly increased for 1% milk ($p=0.002$).

![Graphs showing latency and duration data](image)

**Figure 5.4 Effects of reduced reward on risk-taking behaviour in male $F_{2}[C57BL/6J*CBA/Ca]$ mice.** To further examine the cost/benefit of traversing the PORT task apparatus to collect reward in the presence of a predator odour, we decreased the ‘benefit’ by using a more dilute reward. Reducing the concentration for the reward from 10% to 1% led to significantly increased reward collection latencies, suggesting that the mice were less willing to take the risk of crossing the middle chamber to collect the reward (a). The effect of the rat odour was maintained. In contrast to the effects of predator odour, this effect was not a result of increased emergence times from the start chamber (b), but rather a difference in behaviour in the middle chamber where strain differences in duration persisted (c). Error bars show mean ±SEM. N=13. *$p<0.05$. 
5.3.4 Correlation of independent measures with PORT task behaviour

Independent measures of motivation, motor function and anxiety of \(F_2[C57BL/6J*CBA/Ca]\) mice were further investigated to assess whether general differences in these behaviours could account for the differences shown in the PORT task. To assess whether individual differences in motor activity could have impacted on the behavior in the PORT task, correlations were conducted between the total LMA and the latency to collect the reward in the different predator odour tests. No significant correlations were found when LMA was compared to the latency to collect the reward in the cat odour test of the PORT task (Fig. 5.5a; \(r_{13}=-0.13, p=0.68\)), nor with comparisons of LMA with collection latency on rat \((r_{13}=-0.43, p=0.14;\) data not shown) or fox \((r_{13}=-0.01, p=0.97;\) data not shown) odour tests, suggesting that individual levels of locomotion were having no effect on risk-taking behaviour per se. Correlations between the final reward preference obtained from the reward habituation test and the latency to collect the reward in the different predator odour tests was also not significant for cat odour (Fig. 5.5b; \(r_{13}=0.05, p=0.89\)), rat odour \((r_{13}=-0.17, p=0.58;\) data not shown) or fox odour \((r_{13}=0.01, p=0.99;\) data not shown); suggesting that levels of general motivation for reward also did not impact on PORT task performance, and that in all of the mice there was an equal drive to collect the reward.

Overall, \(F_2[C57BL/6J*CBA/Ca]\) mice showed the expected anxiogenic pattern of behaviour of avoidance of the open arms on the EPM test, making less entries (Fig. 5c; main effect of ARM, \(F_{1,12}=82.50, p<0.001\)) and spending less time in the open arms (Fig. 5d; Wilcoxon signed ranks test, \(Z =-3.18, p<0.005\)). To assess whether individual differences in anxiety-related behaviours impacted on the behavior in the PORT task, correlations were conducted between the proportion of time spent on the open arm of the EPM and the latency to collect the reward in the PORT task. There was a significant positive correlation between time on the open arm of the EPM and the reward latency if cat odour was present in the PORT task (Figure 5.5e; \(r_{13}=-0.58, p=0.04\)), similar significant correlations were observed in comparison with the rat \((r_{13}=-0.645, p=0.017;\) data not shown) and fox \((r_{13}=0.695, p=0.008;\) data not shown) odour tests, further suggesting that inherent anxiety played a significant role in the risk-taking behaviour assessed by the PORT task.
Figure 5.5 Measures of locomotor activity, motivation and anxiety in male $F_2$[C57BL/6J*CBA/Ca] mice. To further evaluate the effects of the PORT task testing, a number of ancillary assessments were performed which investigated motor activity, motivation and anxiety. Total activity levels were not significantly correlated with the latency to collect reward in the PORT task with cat odour present (a); suggesting that motor competence did not affect PORT task performance. Similarly, overall reward preference did not correlate with the latency to collect reward in the PORT task with cat odour present (b). Anxiety-related behaviour was assessed using the elevated-plus maze, which measured entries on to (c) and time spent on (d) the open and closed arms. Results showed a significant negative correlation between open arm times and the latency to collect reward in the PORT task with cat odour present (e), which suggests that inherent anxiety played a significant role in the risk-taking behaviour assessed by the PORT task. For each of the correlations presented here, similar patterns were observed in comparison with the latency to collect the reward in the PORT task during the rat and fox odour tests. Error bars show mean±SEM. N=13. ***P<0.005.

5.3.5 Predator odour induces changes in startle response

To investigate if the $F_2$[C57BL/6J*CBA/Ca] mice found the predator odour anxiogenic, we measured the acoustic startle response (ASR) and prepulse inhibition (PPI) following exposure to either clean bedding or bedding containing fox odour (10% TMT). The mice demonstrated elevated startle responding following exposure to the fox odour
compared to exposure to control bedding (Fig. 5.6a; main effect of ODOUR, $F_{1,12}=14.78$, $p<0.005$). Levels of PPI were generally unaffected by pre-exposure to the fox odour (Fig. 5.6b; main effect of ODOUR, $F_{1,12}=1.02$, n.s.) and differing prepulse intensities induced increasing amounts of PPI (Fig. 5.6c; $F_{2,24}=30.11$, $p<0.001$), although there was a flattening of the PPI effect at the highest prepulse intensity (16 db) used (interaction between Odour*PPI, $F_{2,24}=12.44$, $p<0.001$). Correlations between the mean ASR showed a significant relationship between the amount startle responding following pre-exposure with control (Fig. 5.6d; $r_{13}=0.77$, $p<0.005$), but not fox odour (Fig. 5.6d; $r_{13}=0.45$, n.s) and the latency to collect reward in the PORT task with cat odour present. No such significant correlations were found between PPI (at 16 db prepulse amplitude) and the latency to collect reward in the PORT task with cat odour present (Fig. 5.6e; $r_{13}=-0.56$, and $r_{13}=0.05$, n.s.) for control and fox odour pre-treatment respectively).
Figure 5.6 Effects of predator odour on the acoustic startle response and prepulse inhibition in male $F_2$[C57BL/6J*CBA/Ca] mice. It has been shown previously that the acoustic startle response can be increased in the presence of a stress-inducing predator odour, such as TMT from fox urine (Hebb et al., 2003). Exposure to fox odour, compared to control odour, significantly increased responding to 120 db startle stimuli (a), startle amplitude increased with stimulus intensity in both control and fox odour conditions (b). Increasing prepulse intensities led to increased prepulse inhibition (PPI) (c), which were mainly unaffected by pre-exposure to fox odour, although PPI was reduced at the highest prepulse stimulus tested. Startle responding to 120 db startle stimuli in control, but not fox odour, pre-exposure conditions significantly correlated with the latency to collect reward in the PORT task with cat odour present (d). PPI with a 16 db prepulse intensity in either test condition did not correlate with the latency to collect reward in the PORT task with cat odour present (e). For each of the correlations presented here, similar patterns were observed in comparison with the latency to collect the reward in the PORT task during the rat and fox odour tests. These results suggest that mice showed stress-related patterns of behaviour after exposure to a predator odour. Error bars show mean±SEM. N=13. ***p<0.005.
5.4 Discussion

The principal aim of this chapter was to develop a novel murine test of risk-taking behaviour, which provides a more ethologically valid method than established tests of risk-taking. By exploiting the innate fear that mice have of predator odours, we created a situation which caused a trade-off between the benefit of a food reward and the cost of potential danger. A cohort of WT mice with mixed genetic background (F$_2$[C57BL/6J*CBA/Ca]), chosen as it provides the background strain for Nesp$^{m/+}$ and Grb10$^{+/p}$ mice used in the rest of this thesis, were used to demonstrate that the presence of a predator odour systematically increased the time to collect the food reinforcement in the task. A similar effect on latency was produced by decreasing the value of the reward. Taken together these data suggest that the PORT task is sensitive to changes in both the cost (risk of predation) and the benefit (food).

Although a number of rodent tests have successfully measured the equivalent of ‘human’ risk-taking behaviour, such as loss or attenuation of reward as the potential ‘risk’ (de Visser et al., 2011, Ashenhurst et al., 2012, St Onge et al., 2012), they fail to consider the normal risk-taking situation, where there is a trade-off between a benefit and a separate cost. Thus, the current battery of rodent risk-taking tasks may not capture an animal’s ‘real-life’ risky situation. In this novel test of risk for mice, the PORT task, mice were trained to traverse a 3 chambered apparatus to collect a 10% condensed milk reward. On separate test days the subjects were required to pass through the middle compartment in which the odour from mouse predators (rat, cat or fox) had been placed. Under these conditions the overall latency to collect the reward was significantly increased. Furthermore when this measure was examined in detail, it was found that under predator odour conditions, mice spent significantly more time in the start chamber, but not in the middle chamber; suggesting that the increased latency to collect the reward was a result of reluctance to leave the start chamber and not increased exploration of the odour in the middle chamber. In contrast, during test days when their own bedding was present, mice would readily pass through the centre and ‘open’ areas of the arena. The different predator odours had proportionally different effects on latency, such that rat<fox<cat. Given the nature of how these odours were obtained it is difficult to draw any conclusion from this finding, other
than that in general behaviour in the PORT task was sensitive to a number of different predator odours. Observationally, and as evidenced by the EthoVision tracking (Fig 5.3a), it was clear that under predator odour conditions, mice spent significantly more time in the corners of the three arenas and displayed greater levels of thigmotaxis (wall-seeking behaviour). Thigmotaxis is a fear-based reaction, and rodents display this behaviour as a way of avoiding potentially dangerous areas or threats (Choleris et al., 2001).

Converging evidence for the role of fear in mediating behaviour in the PORT task came from measures on the elevated plus-maze (EPM), which correlated with behaviour in the PORT task. Specifically the amount of time spent on the open arms was positively correlated with latency to collect the reward. Furthermore, the presence of fox odour (TMT) produced an increased startle reaction in the acoustic startle response (ASR), consistent with previous findings (Hebb et al., 2003) and demonstrated a fear inducing reaction to the odour, as opposed to a noxious reaction. Taken together these findings indicate that under certain test conditions the PORT task successfully constituted a sufficiently fear evoking environment, in which collecting the reward would be perceived as a risk. In addition to this, general levels of locomotor activity did not correlate with latency to collect the reward, suggesting that the effect found was not caused by general motoric ability. Furthermore, the latency to collect the reward was not correlated with preference for the condensed milk reward, suggesting that all animals regarded the reward as equally valuable, and the effects found in the PORT task were not due to differing motivation for the reward.

Importantly the PORT task is sensitive to manipulation of the motivation for the food reward, whereby when reward value was reduced, risk-taking behaviour also reduced. Under both control and predator odour conditions, subjects took significantly longer to collect a food reward when reduced from a 10% concentration to a 1% concentration of condensed milk, with the effect on collection latency being comparable to the latency to collect the reward in the presence of rat odour. Therefore the behaviour measured in the PORT task was not just a result of anxiety/fear, but also motivation for the reward. Although there was no general correlation between milk preference and behaviour in the PORT task, these findings indicate that this task successfully measured the trade-off between cost and benefit, which defines risk-taking behaviour.
The data collected here provide evidence for the successful development of a novel risk-taking task, encapsulating a naturalistic and ‘real-world’ approach to the measurement of risk-taking behaviour in rodents. The robust results show an effect which has been replicated across a number of predator odours. The task could be very effective in understanding the neurobiology and neurochemistry of risk behaviour, where the serotonergic, noradrenergic and dopaminergic neurotransmitter systems have been strongly implicated (Belzung and Griebel, 2001, Cardinal, 2006, Doya, 2008). Furthermore the PORT task will be important in assessing the Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice, in order to ascertain if this aspect of risk-taking behaviour is altered in these animals. This explicit test of risk-taking will contribute to our understanding of what specific adult behaviours are affected by Nesp and Grb10.

5.4.1 Summary of key results from Chapter 5

- A cohort of WT male F\textsubscript{2}[C57BL/6J\textsuperscript{*}CBA/Ca] mice were used to successfully develop a novel test of risk-taking behaviour. Under control conditions mice readily crossed the central chamber of the PORT apparatus to collect a food reward; however under predator odour conditions (rat, cat, fox) subjects took significantly longer to cross the central chamber to collect the reward.
- It was demonstrated that when the value of the food reward was reduced, risk-taking behaviour was reduced. This provides evidence that the behaviour in the PORT task was a function of balancing motivation for the reward and fear of predation.
- Evidence from ancillary measures (LMA, EPM and ASR) demonstrated that subjects had a fear/anxiety based response to the predator odour and that behaviour in the PORT task was not driven by general levels of locomotion.
Chapter 6 – Investigating risk-taking behaviour in Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice, using the Predator Odour Risk-Taking (PORT) task.

6.1 Introduction

Previous research has suggested that the maternally expressed imprinted gene Nesp may be associated with the promotion of risk-taking behaviour, in that Nesp\textsuperscript{m/+} mice were shown to have altered reactivity to a novel environment in both a locomotor activity task (LMA) and a reduced propensity to explore a novel environment in a novelty place preference (NPP) task (Plagge et al., 2005). Chapter 3 of this thesis showed the testing of Grb10\textsuperscript{+/p} mice on the LMA and NPP tests and found that Grb10\textsuperscript{+/p} do not exhibit altered behaviour in these tests compared to WT animals. However, Nesp\textsuperscript{m/+} mice (Isles et al. unpublished data) and Grb10\textsuperscript{+/p} mice (Chapter 4) were shown to have opposite behaviour in a test of delay discounting, as measured by the delayed-reinforcement task. Specifically, Nesp\textsuperscript{m/+} mice were significantly more likely to discount the large delayed reward in favour of the small reward, whilst Grb10\textsuperscript{+/p} mice demonstrated the opposite behaviour, having a preference for the large delayed reward.

Although more generally recognised as a test of impulsive behaviour, both theoretical (Kalenscher, 2007) and experimental (Hayden and Platt, 2007) evidence has led some to conclude that discounting tasks measure a facet of risk-taking behaviour in animals, with the argument that animals view temporally distant rewards as risky (Kalenscher, 2007). Therefore, the behaviour of Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice in the delayed reinforcement task could be interpreted as suggesting that Nesp acts to promote risk taking and Grb10 acts to promote risk aversion. However the delayed-reinforcement task is based on inter-temporal decision making (choosing between a temporally close or temporally distant reward), and therefore does not measure a cost/benefit analysis, where a potential gain is weighed up against potential harm/danger. Consequently, it was of interest to examine this behaviour in an additional, more explicit, test of risk-taking.

Chapter 5 outlined the development of a novel test of risk taking, the predator odour risk-taking task (PORT), which provides an ethobiologically relevant test in which mice are
faced with the decision to collect a reward under the potential threat of a predator (see also Dent et al. 2013). The PORT task measures the trade-off between two separate factors; an unpleasant or unwelcome occurrence (potential danger), and something rewarding (obtaining food); therefore taxing a more ethobiologically valid type of risk-taking. During the development of the PORT task in F$_2$[C57BL/6J*CBA/Ca] mice (Chapter 5), various ancillary measurements were made in order to ensure that the task accurately measured risk-taking behaviour. These included measures of locomotor activity, anxiety, potentiated startle response and pre-pulse inhibition following exposure to predator odour. Furthermore, the effect of reducing the reward value was assessed, which confirmed that a reduction in reward value increased the latency to collect the reward, during both predator odour and control trials. This suggested that the behaviour measured in the PORT task was not just a result of anxiety/fear, but also motivation for a reward. As a result of this wide range of measurements, we are satisfied that the PORT task successfully measures risk-taking behaviour in rodents, and therefore provides a useful tool which can be applied to transgenic mice in order to assess how certain genes may influence this behaviour.

This chapter investigated Nesp$^{m/+}$ and Grb10$^{+/p}$ mice performance in the PORT task, to analyse if this aspect of risk-taking is mediated by the imprinted genes Nesp and Grb10. Based on previous findings it may be expected that Nesp$^{m/+}$ mice will be less risk-taking, and therefore take longer to collect the reward in the PORT task. Conversely Grb10$^{+/p}$ mice may be expected to be more risk-taking, and therefore be significantly quicker to collect the reward in the PORT task. The results of this task will contribute to our understanding of which specific facets of risk-taking behaviour the imprinted genes Nesp and Grb10 influence.

6.2 Methods

6.2.1 Subjects

Two lines of mice were used: a cohort of Nesp$^{m/+}$ mice and their WT littermates, and separately a cohort of Grb10$^{+/p}$ and their WT littermates. All mice were 6 months old at the start of testing. Details of specific samples sizes per genotype and weights at the start of testing can be found in Table 6.1. All subjects were group housed (2 to 5 mice/cage) in
environmentally enriched cages (i.e. with cardboard tubes, shred-mats and tissue paper) in a temperature and humidity controlled *vivarium* (21 ± 2°C and 50 ± 10%, respectively) with a 12-hour light-dark cycle (lights on at 07:00 hours/lights off at 19:00 hours). Animals had *ad libitum* access to standard lab chow, but home cage water was restricted to 2 hour access per day during the period of testing (access occurred after testing). This regime maintained the subjects at ~90% of free-feeding body weight. Further details of animal husbandry and handling prior to testing can be found in Chapter 2, Section 2.3.

<table>
<thead>
<tr>
<th>Nesp cohort</th>
<th>Grb10 cohort</th>
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</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td><strong>Number</strong></td>
</tr>
<tr>
<td>WT</td>
<td>17</td>
</tr>
<tr>
<td>Nesp\textsuperscript{m/+}</td>
<td>19</td>
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<tr>
<td>WT</td>
<td>14</td>
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<tr>
<td>Grb10\textsuperscript{+/p}</td>
<td>11</td>
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<tr>
<td><strong>Average weight (g)</strong></td>
<td><strong>Average weight (g)</strong></td>
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<td>29 (±2)</td>
<td>30 (±3)</td>
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<tr>
<td>35 (±4)</td>
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*Table 6.1 Details of the sample sizes and average weights of each cohort used in PORT task.*

6.2.2 Predator odour risk taking task

The predator odour risk-taking (PORT) task was carried out using the 3-chamber apparatus as shown in Chapter 2, Section 2.4.6, and procedure as described in Chapter 5, Section 5.2. The two cohorts of mice were tested separately but consecutively within the same period of time, such that the *Nesp* cohort completed the task during the 1st week, then the *Grb10* cohort completed the task the following week. During the development of the PORT task (see Chapter 5, Section 5.2.3) an array of predator odours were used (rat, cat and fox), and the result of these experiments showed that fox odour (synthetic TMT) was highly effective in inducing robust behavioural effects, and was also the most controllable and quantifiable (due to the nature of it being a synthetic chemical). The fox odour also gave robust potentiated startle response effects (Chapter 5, Section 5.3.5); therefore only this odour was used for testing *Nesp*\textsuperscript{m/+} and *Grb10*\textsuperscript{+/p} mice in the PORT task, in comparison to control tests using the subjects own bedding.

6.2.2.1 Habituation and Acquisition

Details of the habituation and acquisition stages of the PORT task are outlined in Chapter 5 section 5.2.4, but in brief; both cohorts of animals were first habituated to the
condensed milk reward using the milk preference test protocol as outlined in Chapter 2, Section 2.3.5. Subjects were then given a single 20 minute period of habituation to the apparatus, in which they were given the opportunity to freely explore all three chambers of the apparatus without any reward or odour stimuli present. Following habituation, each subject completed the acquisition stage; consisting of 5 consecutive trials, in which mice were placed in the apparatus and required to cross the central chamber, containing clean bedding, in order to collect the reward available in the opposite chamber. These stages of the task allowed subjects to become familiar with the apparatus, and learn that a reward was available. During the 5 acquisition trials, the latency to collect the reward was recorded; as determined from analysis of the tracking data collected by EthoVision tracking software (see Chapter 2, Section 2.4.7). Subjects had a maximum of 10 minutes to collect the reward.

6.2.2.2 Predator odour risk testing

When subjects had successfully completed the habituation and acquisition stages, they moved on to the PORT task proper. Trials were carried out in the same way as previously described in Chapter 5, Section 5.2.4.4, in brief; subjects were individually put in either the left or right chamber (counterbalanced across subjects) and required to cross the middle chamber (containing bedding) in order to collect the reward (10% condensed milk solution) located in the opposite chamber (see Chapter 5, Figure 5.1). Subjects completed 3 consecutive ‘control’ trials (where clean bedding was present) in the morning, and 3 consecutive ‘test’ trials in the afternoon (where bedding containing an odour was present). The odours tested in this experiment were self odour (bedding from home cage) and fox odour (TMT). As previously, the main parameters assessed in each trial were the latency to reach the reward chamber and collect the reward from the start of each trial, the duration of time spent in each chamber, the distance moved, movement velocity, and the amount of rearing in each chamber.

6.2.3 Data analysis and statistics

For each of the different test odour sessions (control bedding, self and fox odours), mean values were determined by averaging each of the 3 probe trials together. The following parameters were analysed for each session: latency to enter the reward chamber
from the start of the trial, duration within the start, middle and reward chambers, distance moved in the start and middle chambers, the amount of rears in the start and middle chambers and the velocity of movement in the start, middle and reward chambers. Data comparing the effect of predator odour on behaviour was analysed by ANOVA with repeated measures of ODOUR (self and fox) and CHAMBER (start or middle). Habituation data was analysed by ANOVA with a repeated measures of TIMEBIN (0-5, 6-10, 11-15, 16-20) and CHAMBER, acquisition data with repeated measures of TRIAL (1-5) and CHAMBER and habituation to the reward by ANOVA with factors of DAY (1-6) and solution (water, 10% condensed milk). Pearson’s correlations were used to assess relationships between key parameters from each of the different tests. For repeated-measures analyses, Mauchly’s test of sphericity of the covariance matrix was applied. Huynh-Feldt corrections were applied as necessary, and adjusted degrees of freedom are provided. Data presented are mean ± SEM. All statistical analyses were carried out using SPSS version 18.0.

6.3 Results

6.3.1. Nesp\textsuperscript{m/+}

6.3.1.1 Milk preference testing and acquisition of PORT task

Before subjects could be assessed for risk-taking behaviour in the PORT task, they were first habituated to the 10% condensed milk reward solution. Habituation to the condensed milk reinforcer showed that all mice had a significant preference for the condensed milk reward solution over water, reaching >60% preference by the end of the habituation test (Fig 6.1a; main effect of SESSION $F_{4,136}=28.02$, $p<0.001$, main effect of GENOTYPE $F_{1,34}=1.10$, $p=0.30$), consuming an average 1.2 ml of solution in the final session. Subjects were then habituated to the test apparatus in a single 20 minute session. During the 20 minute habituation session, all mice readily explored the 3-chamber apparatus, making more entries into the middle chamber (Fig 6.1c; main effect of CHAMBER, $F_{2,68}=5.46$, $p<0.01$) as might be expected, as the mice traversed the apparatus, which was also reflected by an increase in the amount of time spent in this chamber (Fig 6.1b; main effect of CHAMBER, $F_{2,68}=640.83$, $p<0.001$). Following the habituation stage, subjects underwent
training to acquire the basic procedure of exiting the start chamber, traversing the middle chamber, and collecting the reward in the furthermost chamber. During acquisition of the PORT task procedure, the mice were trained, across 5 trials, to traverse the apparatus from the start chamber to collect 100 μl of 10% condensed milk in the reward chamber, traversing the middle chamber which contained clean standard (control) bedding. All subjects spontaneously exhibited this behaviour, and readily collected the reward in a relatively quick time, whereby both Nespm/+ and WT mice showed an average latency of under 60 seconds on the first trial. The time taken to collect the reward subsequently increased with trial number in all mice (Fig 6.1d; main effect of SESSION, F4,136=17.45, p<0.001: main effect of GENOTYPE, F1,34=0.88, p=0.36), a pattern of behaviour which was also seen in the F2[C57BL/6J*CBA/Ca] mice during the development of the PORT task (Chapter 6, Figure 6.2).
Figure 6.1 Reward habituation and acquisition of the PORT task procedure by Nesp<sup>mr/+</sup> mice. During habituation to the reward, all mice showed an increasing preference for the 10% condensed milk reward over water (a). During the 20 min habituation session to the 3-chamber apparatus all mice readily explored the different chambers of the PORT task apparatus, spending most time in the middle compartment (b), but entering each compartment a number of times (c). During acquisition, the mice were trained to cross the PORT task apparatus to collect the reward, passing through the middle chamber which now had clean bedding material on the floor. All the mice traversed the chambers quite rapidly (d). Data shows mean±SEM.

**P<0.01, ***P<0.005.

6.3.1.2 Behaviour in PORT task

Following acquisition, subjects were assessed for risk-taking behaviour by mixing the bedding material in the middle chamber of the PORT task apparatus with either their own odour, or fox (TMT) odour. Control sessions were also carried out, with clean untainted bedding. Mice were given 3 training trials with control bedding in the central chamber in the
morning and then 3 test trials with bedding mixed with a control (own bedding) or fox odour in the afternoon. For ease of viewing the 3 trials of each test session have been collapsed to yield single values.

In the presence of a predator odour all mice were more reluctant to collect the food reward relative to control (own) odour as evidenced by the overall latencies to collect the reward (Fig. 6.2a; main effect of ODOUR, $F_{1,34}=31.66, p<0.001$). In the presence of unthreatening odour (i.e. own or clean odour) mice would readily cross the central chamber in order to collect the reward. Under predator odour conditions $Nesp^{m/+}$ mice appeared to take longer than WT controls to collect the reward, however this finding did not reach significance (Fig. 6.2a; main effect of GENOTYPE, $F_{1,34}=0.59, p=0.49$). A breakdown of the overall duration indicated that the presence of fox odour significantly increased the time spent in the start chamber (Fig. 6.2b; main effect of ODOUR, $F_{1,34}=15.72, p<0.001$, main effect of GENOTYPE, $F_{1,34}=0.81, p=0.36$) but not the time spent in the middle chamber (Fig. 6.2c; main effect of ODOUR, $F_{1,34}=3.89, p=0.06$, main effect of GENOTYPE, $F_{1,34}=0.04, p=0.85$) across all subjects.

The Latency to collect the reward in the port task did not correlate with the condensed milk preference (on final day of testing) during either fox odour ($r_{36}=-0.03$, n.s. data not shown) or self odour ($r_{36}=0.17$, n.s. data not shown) trials. This provided evidence that the behaviour shown in the PORT task was not driven by decreased preference for the reward alone.
6.3.2 Grb10+/p

6.3.2.1 Milk preference testing and acquisition of PORT task

As with the Nesp\textsuperscript{m/+} cohort habituation to the condensed milk reinforcer showed that all mice had a significant preference for the condensed milk reward solution over water, reaching >60% preference by the end of the habituation test (Fig 6.3a; main effect of SESSION, $F_{4,92}=24.71$, $p<0.001$, main effect of GENOTYPE, $F_{1,23}=0.01$, $p=0.91$), consuming an
average 1.0 ml of solution in the final session. During the 20 minute habituation session, mice readily explored the 3-chamber apparatus, making more entries into the middle chamber (Fig 6.3c; main effect of CHAMBER, $F_{2,46}=132.88, p<0.001$), as might be expected as the mice traversed the apparatus, which was also reflected by an increase in the amount of time spent in this chamber (Fig 6.3b; main effect of CHAMBER, $F_{2,46}=929.67, p<0.001$). During acquisition of the PORT task procedure, the mice were trained, across 5 trials, to traverse the apparatus from the start chamber to collect 100 μl of 10% condensed milk in the reward chamber, traversing the middle chamber which contained clean standard (control) bedding. The time taken to collect the reward increased with trial number (Fig 6.3d; main effect of SESSION, $F_{4,64}=22.99, p<0.001$) cohorts. Both $Grb10^{+/p}$ and WT mice showed similar times to collect the reward (Fig 6.2d; main effect of GENOTYPE, $F_{1,16}=0.39, p=0.54$).
Chapter 6 | Investigating risk-taking behaviour in *Nesp*<sup>−/−</sup> and *Grb10*<sup>+/−</sup> mice, using the Predator Odour Risk-Taking (PORT) task

6.3.2.2 Behaviour in PORT task

Generally the *Grb10* cohort (both *Grb10*<sup>+/−</sup> and WT mice) were slower to collect the reward in the PORT task than the *Nesp* cohort, but showed an analogous pattern of behaviour. Whereby in the presence of a predator odour, all mice were more reluctant to
collect the food reward relative to control (own) odour as evidenced by the overall latencies to collect the reward (Fig. 6.4a; main effect of ODOUR, $F_{1,23}=21.66$, $p<0.001$). In the presence of unthreatening odour (i.e. own or clean odour) mice would readily cross the central chamber in order to collect the reward. Under predator odour conditions $Grb10^{+/p}$ mice appeared to be quicker to collect the reward than WT controls, however this finding also did not reach significance (Fig. 6.4a; main effect of GENOTYPE, $F_{1,23}=3.05$, $p=0.09$).

A breakdown of the overall duration to collect the reward revealed that the presence of fox odour significantly increased the time spent in the start chamber (Fig. 6.4b; main effect of ODOUR, $F_{1,23}=15.60$, $p<0.005$, main effect of GENOTYPE, $F_{1,23}=1.87$, $p=0.19$), as well as the time spent in the middle chamber (Fig. 6.4c; main effect of ODOUR, $F_{1,23}=6.48$, $p<0.05$, main effect of GENOTYPE, $F_{1,23}=0.40$, $p=0.54$) across all subjects. Furthermore the latency to collect the reward in the port task did not correlate with the condensed milk preference (on final day of testing) during either fox odour ($r_{25}=0.62$, n.s. data not shown) or self odour ($r_{25}=-0.03$, n.s. data not shown) conditions. This provided evidence that the behaviour shown in the PORT task was not driven by increased preference for the reward alone.
Figure 6.4 Effects of different predator odours on risk-taking behaviour in Grb10\textsuperscript{+/p} mice. Mice were assessed for the time taken to cross the PORT task apparatus in the presence of different odour/bedding material combinations, including their own given bedding. Mice were given 3 control trials and 3 test trials in each session, for ease of viewing, the 3 trials in each phase have been collapsed to yield single values. Overall, mice were slower to collect the reward in the presence of a predator odour (a). Increased latencies to collect the reward were mainly as a result of increased emergence times from the start chamber (b), as well as increased time in the middle chamber (c). Data shows mean±SEM.

*P<0.05, ***P<0.005.
6.4 Discussion

The principal aim of this chapter was to assess the effect the imprinted genes *Nesp* and *Grb10* have on risk-taking behaviour; using a novel predator odour risk-taking task (PORT), which provides a more ethologically valid method than established tests of risk-taking (Dent et al., 2013). Two lines of genetically modified mice, *Nesp*<sup>m/+</sup> and *Grb10*<sup>r/p</sup> along with their respective littermate controls, were assessed using the PORT task. In the presence of a predator odour, both cohorts systematically increased the time taken to cross the central chamber and collect the food reinforcement, demonstrating an attenuation of risk-taking behaviour in the presence of fox odour compared to control conditions. However, no significant differences in risk-taking behaviour were seen between *Nesp*<sup>m/+</sup> and their WT littermate controls, and *Grb10*<sup>r/p</sup> and their WT littermate controls.

The data collected here provided evidence for the successful application of a novel risk-taking task, whereby the previous effects found in cohort of F<sub>2</sub>[C57BL/6J*CBA/Ca] mice (Chapter 5) have been replicated in two further mouse lines. Both lines of mice showed a similar progression through the task, showing equivalent amounts of time spent in each chamber during habituation (Fig. 6.1b and 6.2b) and the same pattern of behaviour in terms of latency to collect the reward during the acquisition stage (Fig. 6.1d and 6.2d). During the task proper, the *Nesp* cohort demonstrated quicker overall levels to collect the reward, than the *Grb10* cohort, across control and fox odours; this is likely to be due to the fact that the two lines are maintained on slightly different genetic backgrounds. Independent measures of motivation for the food reward were analysed, and correlated with subjects’ performance in the PORT task. Results indicated that both *Nesp*<sup>m/+</sup> and *Grb10*<sup>r/p</sup> subjects, and their respective WT subjects had equal motivation for the condensed milk and that preference for this reward was not correlated with the time taken to collect the reward in the PORT task when predator odour was present. Therefore the behaviour found in the PORT task was not a result of differing motivation for the food reinforcement.

The data collected here provided evidence for the application of the PORT task to transgenic mouse models. Whereby subjects in two separate transgenic lines successfully learnt the task and exhibited altered behaviour under the threat of predator odour,
demonstrated by significant increases in the latency to collect the reward. The results of the PORT task revealed no significant differences between transgenic mice (Nesp$^{m/+}$ or Grb10$^{+/p}$) and their respective WT littermates, in risk-taking behaviour. Based on this finding, it can be concluded that these genes do not influence this aspect of risk-taking and that perhaps delay-discounting and the PORT task channel separate neurological systems that control these aspects of risk-taking behaviour.

Whilst the results of the PORT task did not yield any significant differences between Nesp$^{m/+}$ or Grb10$^{+/p}$ and their respective WT controls, it is interesting to note that the direction of the trends in the PORT task were opposite for Nesp$^{m/+}$ and Grb10$^{+/p}$ mice, and in a direction that is consistent with previous results. The Nesp$^{m/+}$ mice showed more risk-averse behaviour (slower to collect the reward), and Grb10$^{+/p}$ mice showed more risk-taking behaviour (quicker to collect the reward). The trends demonstrated in the genetically altered animals, mimic the effects found in previous experiments. It was previously demonstrated that Nesp$^{m/+}$ animals showed increased delay-discounting behaviour, whilst Grb10$^{+/p}$ showed decreased delay-discounting behaviour, in the delayed reinforcement task (outlined in Chapter 4). The atypical behaviour in the delay-discounting task, as well as altered reactivity to novel environments in the LMA, OF and NPP tasks, suggests that Nesp$^{m/+}$ mice could be interpreted as having increased risk aversion, whilst results from the delayed-reinforcement task suggest that Grb10$^{+/p}$ mice could be interpreted as having increased risk-taking behaviour. It would therefore be valuable to examine Nesp$^{m/+}$ or Grb10$^{+/p}$ mice in the PORT task with an increased number of subjects, in order to observe if increased power would yield a significant difference in risk-taking behaviour in this task.

The use of the PORT task in conjunction with pharmacological manipulation may provide another way to elucidate if Nesp and Grb10 do have effects on risk-taking behaviour in this task. Expression of Nesp and Grb10 in the locus coeruleus and dorsal raphe nucleus (Plagge et al., 2005, Garfield et al., 2011) suggests that these genes may exert their influence on risk-taking behaviour via the noradrenergic and/or serotonergic system. Furthermore expression of Grb10 in the ventral tegmental area and substantia nigra pars compacta may also imply that this gene exerts influence on behaviour via the dopaminergic system (Garfield et al., 2011). Therefore by using drugs that target the monoaminergic systems, it may be possible to find a differential effect on behaviour in Nesp$^{m/+}$ and Grb10$^{+/p}$.
mice in the PORT task. However the nature of the PORT task means it is vulnerable to the effects of habituation as the potential threat of a predator attenuates over time, meaning that repeated measures experimental designs are not prohibitive (Dent et al., 2013). Therefore the PORT task is less amenable to drug studies which are useful for understanding the neurobiological underpinnings of risk-taking behaviour (Zeeb et al., 2009). However a drug study using a within-subjects experimental design could elucidate any potential differences Nesp and Grb10 may have on this aspect of risk-taking behaviour.

The delay-discounting task is based in a highly controlled but ethologically artificial environment, whilst the PORT task is based in an ‘ethologically plausible semi-naturalistic environment’ (Choi and Kim, 2010). Therefore the additional contextual information provided in the PORT task, as well as the heightened involvement of affective processes (i.e. the increased emotion of the fear response to the predatory threat) may cause different neurological processes to control this behaviour. For example the Amygdala is the crucial brain structure for regulating emotion/fear (Blanchard and Blanchard, 1972), and plays a pivotal role in mediating risk-taking behaviour under fear conditions in environments such as that used in the PORT task (Choi and Kim, 2010). Therefore the risk-taking behaviour in the PORT task may be controlled by different neurological structures to risk-taking behaviour in operant chamber-based tasks. Consequently, another route to further understand the effect Nesp and Grb10 have on risk-taking behaviour would be to test Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice on operant tasks of risk-taking, such as rodent analogues of the Iowa gambling task (Zeeb et al., 2009) and the balloon analogue risk-taking task (BART) (Jentsch et al., 2010).
### 6.4.1 Summary of key results from Chapter 6

- The PORT task was successfully applied to two separate transgenic mouse lines, to examine risk-taking behaviour in $Nesp^{m/+}$ and $Grb10^{+/p}$ mice.

- All subjects demonstrated a significant change in behaviour when exposed to predator (fox) odour compared to control conditions, whereby presence of fox odour increased the latency to collect the reward.

- $Nesp^{m/+}$ and $Grb10^{+/p}$ mice showed no significant difference to WT controls in latency to collect the reward in the PORT task, suggesting that $Nesp$ and $Grb10$ do not influence this aspect of risk-taking behaviour.
Chapter 7 – Histological investigation into Nesp and Grb10

7.1 Introduction

Previous work investigating a knockout mouse model of Nesp (Isles et al, unpublished results), and work from this thesis (Chapter 4) investigating a knockout mouse model of Grb10 has identified delay discounting as behaviour upon which both these genes may impact. Moreover, these oppositely expressed imprinted genes appear to act antagonistically on this behaviour with Nesp"m/+ mice being less tolerant, and Grb10+/p more tolerant, of increasing delays. On a biochemical level, this may suggest that their levels of expression could be inter-related and opposite. The current understanding of the biochemical functions of Nesp55 and Grb10 do not point to an obvious cellular interaction (Ischia et al., 1997, Garfield et al., 2011) therefore to address this, the levels of expression of Nesp and Grb10 in the knock-out models of the opposite gene were investigated to establish if the absence of expression of one of the genes significantly affected expression of the other, and vice versa.

As outlined in the General Introduction, previous studies have demonstrated that these two genes show discrete patterns of expression in the mouse brain (Plagge et al., 2005). Furthermore expression of Nesp and Grb10 appears to be highly overlapping in some specific hindbrain nuclei: Edinger-Westphal, dorsal raphe (DRN) and the locus coeruleus (LC), and in the hypothalamus (Figure 7.1), although investigations to date have not demonstrated if the expression of each gene are co-localised in the same cells. Analysis of tissue from adult Grb10+/p mice has revealed that Grb10 is co-localised with the key monoamine neurotransmitters (Garfield et al., 2011), specifically serotonin (5-HT) in the DRN, dopamine (DA) in the substantia nigra pars compacta (SNC), and also acetylcholine (ACh) in the caudate putamen (Figure 7.2), which as outlined in the General Introduction, may provide a link between Grb10 and the regulation of impulsivity and risk-taking behaviours. Although the expression of Nesp within the rodent brain has been previously described with high levels of expression in the hypothalamus, pons, DRN and LC (Bauer et al., 1999, Plagge et al., 2005), it is not clear whether this gene is expressed in 5-HT or noradrenaline positive cells found in these regions.
**Figure 7.1 Schematic of the expression of Nesp and paternal Grb10 in the mouse brain.** Coronal sections (most anterior shown at the top, most posterior shown at the bottom) showing regions where both Nesp and paternal Grb10 are co-expressed are highlighted in purple, including the hypothalamus (HYP), Edinger–Westphal nucleus (EWN), dorsal raphe nucleus (DRN), and locus coeruleus (LC). Regions where paternal Grb10 is expressed only are highlighted in blue, specifically the ventral tegmental area (VTA) and substantia nigra pars compacta (SNC). These latter brain regions are recognised as ‘hotspots’ of imprinting in the brain. *Figure taken from Dent and Isles, 2013.*

**Figure 7.2 Histological sections of Grb10+/p adult brain tissue showing co-localisation of Grb10 with monoamine neurotransmitters and acetylcholine.** Immunofluorescence staining for β-gal, expressed from the Grb10 paternal allele, with markers specific for (a) dopaminergic neurons within the substantia nigra pars compacta, identified with the dopamine transporter (DAT), (b) serotonergic neurons within the dorsal raphe nucleus, identified with a marker for serotonin (5-HT), and (c) cholinergic inter-neurons within the caudate putamen, identified with the choline acetyltransferase (ChAT). Arrows indicate cells with co-localisation of staining of these markers and β-gal, used as the marker for Grb10. *Figure taken from Garfield et al. 2011.*
Therefore the aims of this series of experiments was, firstly, to investigate the reciprocity of gene expression in \(\text{Grb10}^{+/p}\) and \(\text{Nesp}^{nm/+}\) mutant mice using qPCR; secondly, to expand upon the previous findings that \(\text{Grb10}\) and \(\text{Nesp}\) are co-expressed in the same brain regions. Immunofluorescence was used to investigate if Nesp55 and Grb10 are expressed in the same cells of these areas; and finally, whether Nesp55 co-localises in 5-HT or noradrenergic neurones of the DRN and LC, respectively, consistent with the previous results found with Grb10 (Figure 7.2, Garfield et al., 2011).

7.2 Methods

7.2.1 qPCR analysis of expression

Brains were removed from adult \(\text{Grb10}^{+/p}\) mutant mice, \(\text{Nesp}^{nm/+}\) mutant mice and their respective wild-type (WT) littermates. Whole brains were hand-dissected to remove the hypothalamus and the midbrain. RNA was extracted and prepared according to the protocol previously described (Chapter 2, Section 2.5.2.2). cDNA samples were then made using Clontech® RNA to cDNA (Oligo dT) kit for PCR, according to the protocol in Chapter 2, Section 2.5.2.6. Real-time quantitative PCR (qPCR) was then carried out using optimised primers specific to \(\text{Nesp}\) and \(\text{Grb10}\), to assess gene expression levels relative to a housekeeping gene (\(18S\) rRNA). All reactions were carried out in 72-well GeneDisk® in triplicates to minimize pipetting error, using a robotic liquid handling system (Corbett Life Science, CAS-1200); reactions were also carried out alongside NTC (Non-Template Control) samples containing no RNA. Samples were then run on the Rotor-Gene™6000 qPCR machine (Corbett Life Science) according to the manufacturer’s specifications. \(18S\) was used as the housekeeping gene to which expression data of \(\text{Nesp}\) and \(\text{Grb10}\) were normalised to using the \(\Delta\Delta\text{Ct}\) method, as described in Chapter 2, Section 2.5.2.9. Acquired data were transformed \((2^{\Delta\Delta\text{Ct}})\) and statistically analysed using a two-tailed t-test.
7.2.2 Immunohistochemistry and Immunofluorescence

7.2.2.1 Preparation of tissue

Grb10^{+/p} mutant mice, plus their WT littermates, and Nesp^{m/+} mutant mice and their WT littermates were perfused as outlined in Section 2.5.3.1. Whole brains were then dissected and sectioned into 40 μm coronal slices using a freezing microtome, according to the protocol outlined in Section 2.5.3.2.

7.2.2.2 Antibodies

The Grb10^{+/p} mutant mouse strain is derived by insertion of a LacZ:neomycin<sup>+</sup> gene-trap cassette within Grb10 exon 8 (as described in Garfield et al. 2011), therefore transmission of the Grb10KO allele separately through the paternal line generates heterozygous progeny in which the paternal Grb10 allele is disrupted by the β-geo cassette (Grb10^{+/p}). Therefore, the LacZ reporter gene is expressed in place of paternal Grb10. As a proxy for Grb10 a β-galactosidase (β-gal) specific antibody was used as previously (Garfield et al., 2011). The Nesp55 primary antibody (generated and obtained from Prof. R. Fischer-Colbrie Lab, Innsbruck Medical University, Austria) is a rabbit anti-Nesp55 polyclonal antibody, recognizing the free terminal end (GAIPRRH) of Nesp55. It has been successfully characterised and used previously (see Ischia et al. 1997) at a 1:1000 dilution. The batch acquired was obtained from a new bleed and therefore required characterising. For immunohistochemistry, a biotinylated secondary antibody (as part of the Vectastain<sup>®</sup> Elite ABC Kit) was used. All antibodies were optimised (with the exception of anti-Nesp55), using 3 relevant dilutions of the antibodies (based on recommended dilution), in order to determine the optimal dilution. The antibodies used for immunofluorescence are detailed in Table 7.1.
Table 7.1 Primary and secondary antibodies used for immunofluorescence analysis

7.2.2.3 Immunohistochemistry

For the Immunohistochemistry of brain sections from \( Nesp^{m/+} \) and WT littermates a Vectastain® Elite ABC Kit (PK-6100) was used. Sections were first washed in TBS (4 x 10 minutes). They were then incubated in a peroxidase block on a stirrer for 30 minutes (0.6% hydrogen peroxidase in TBS) to block endogenous peroxidase. Sections were washed in TBS again (3 x 10 minutes in TBS), and then incubated in TBST with 3% normal goat serum (NGS) (S-1000, vector labs) on a stirrer for 30 minutes at room temperature. Following NGS blocking, sections were incubated in primary antibody (anti-Nesp55) diluted at 1:1000 in TBST with 3% NGS. This was stirred for 10 minutes and then covered and stored overnight at 4°C. The following day the sections were washed 3 x 10 minutes in TBST with 3% NGS. Sections were then incubated for 1 hour in the secondary antibody diluted 1:200 in TBST with 3% NGS at room temperature. Following the secondary antibody incubation sections were washed 3 x 10 minutes in TBST, and then allowed to incubate in the ABC complex (made as per kit specifications) at room temperature, on a stirrer for 1 hour. Sections were then washed as before (3 x 10 minutes in TBST), then washed 2 x 10 minutes in 0.05 M Tris buffer. Sections were then incubated in DAB solution for 35 seconds at room temperature; they were then immediately washed in cold PBS in order to stop the reaction. Finally sections were then washed in TBST for 2 minutes and left in a new change of TBST solution overnight. The following day sections were mounted on polysine coated slides, and allowed
to dry overnight. The mounted slides were then dehydrated through a process of incubation in a rising concentration of alcohol followed by xylene, then cover-slipped and sealed using DPX (Raymond Lamb DPX), and allowed to dry overnight. All experiments were carried out alongside negative controls.

7.2.2.4 Immunofluorescence

Dual-labelling immunofluorescence of Nesp55 with 5-HT/TH was carried out in WT free-floating sections alongside Nesp<sup>m/+</sup> sections as a control. Dual-labelling immunofluorescence analysis of Nesp55 co-localisation with Grb10 (β-gal) was carried out on Grb10<sup>+/p</sup> sections, in order to stain for the Lac-Z reporter gene which is expressed in place of Grb10. Sections were washed three times for 10 min each in 0.1% PBS before being incubated for 15 minutes in 0.3 M glycine in 0.1% PBS at room temperature, to neutralise endogenous aldehyde groups. Sections were washed, as before, in 0.1% PBS and then incubated at room temperature for 1 hour in 10% blocking solution; 0.5% BSA (BB International, Cardiff, UK), 0.5% Triton X-100 (v/v, Sigma Aldrich) in 0.1% PBS. Sections were then transferred to a solution containing the relevant concentration of primary antibody diluted in a 1% blocking solution (dual labelling was achieved by incubation with both primary antibodies of interest); this was allowed to incubate overnight at 4°C whilst gently shaking. The next day sections were washed three times for 10 minutes in 0.1% PBS. The relevant fluorescent secondary antibodies (Alexa Fluor; Life technologies) were diluted 1/1000 in 1% blocking solution, sections were incubated in this solution in the dark at room temperature for 2 hours, whilst gently shaking. Sections were then washed in 0.1% PBS as before (in the dark) and transferred to polysine coated slides and allowed to dry over-night in a dark dust-free environment. The mounted slides were then dehydrated through a process of incubation in a rising concentration of alcohol, followed by xylene, then cover-slipped and sealed using DPX (Raymond Lamb DPX), and allowed to dry over-night. To control for non-specific binding of the secondary antibodies, secondary-only negative controls were carried out alongside all experiments.
7.2.2.5 Image analysis

Immunohistochemistry slides were viewed and images captured using an upright light microscope (Leica DMRB). Immunofluorescence slides were viewed and images captured using an upright fluorescence microscope (Leica DM5000 B). Dual-labelled immunofluorescence images were acquired through separate channels for different wavelengths (488 and 568 nm) then subsequently merged using ImageJ (Image>colour>merge channels).

7.3 Results

7.3.1 qPCR analysis of reciprocal expression of Nesp and Grb10

7.3.1.1 Nesp expression in Grb10+/p and WT littermate brain

qPCR was used to quantify the expression levels in the midbrain and hypothalamus of Nesp in Grb10+/p and WT mice. The melting profiles of 18S and Nesp are shown in Figure 7.3; each reaction was carried out in triplicate therefore values were obtained by collapsing across the triplicates to yield an average value for each sample. The results indicate that there were no differences in the amount of Nesp expression in either the midbrain (Figure 7.4a; $t_{13}=-0.99$, $p=0.35$) or hypothalamus (Figure 7.4b; $t_{13}=1.26$, $p=0.34$) between Grb10+/p and WT mice.
Figure 7.3 Melt curves for housekeeping gene 18S and gene of interest Nesp in Grb10\textsuperscript{+/p} and WT littermate brain. Graphs show the melt curve profiles and melting temperatures for 18S (a) and Nesp (b) in the midbrain and hypothalamus of Grb10\textsuperscript{+/p} (N=9) and WT mice (N=7). Each sample was run in triplicate.

Figure 7.4 Analysis of Nesp expression in the midbrain and hypothalamus of Grb10\textsuperscript{+/p} and WT littermate mice. Expression of Nesp55 was analysed by qPCR and calculated relative to expression of the housekeeping gene 18S. Results show that there were no significant differences in expression of Nesp55 in either the midbrain (a) or hypothalamus (b) of WT (N=7) and Grb10\textsuperscript{+/p} (N=9) mice.
7.3.1.2 Grb10 expression in Nesp<sup>m/+</sup> and WT littermate brain

qPCR was used to quantify the expression levels of Grb10 in Nesp<sup>m/+</sup> and WT littermate mice, in the midbrain and hypothalamus. The melting profiles of 18S and Grb10 are shown in Figure 7.5, and as before, all reactions were carried out in triplicate and final values were obtained by collapsing across the triplicates to yield an average value for each sample. The results indicate that there was no significant differences in the amount of Grb10 expression in either the midbrain (Figure 7.6a; t<sub>8</sub>=-0.01, p=0.99) or hypothalamus (Figure 7.6b; t<sub>9</sub>=-2.23, p=0.05) between Nesp<sup>m/+</sup> and WT mice.

![Figure 7.5 Melt curves for the housekeeping gene 18S and gene of interest Grb10 in Nesp<sup>m/+</sup> and WT littermate brain.](image-url)

Graphs show the melt curve profiles and melting temperatures for 18S and Grb10 in the midbrain and hypothalamus of Nesp<sup>m/+</sup> (N=7) and WT mice (N=5). Each sample was run in triplicate.
Figure 7.6 Analysis of Grb10 expression in the midbrain and hypothalamus of Nesp\textsuperscript{m/+} and WT littermate mice. Expression of Grb10 was analysed by qPCR and calculated relative to expression of the housekeeping gene 18S. Results show that the expression of Grb10 was not significantly different in either the midbrain (a) or hypothalamus (b) of Nesp\textsuperscript{m/+} (N=7) and WT (N=5) mice.

7.3.2 Immunohistochemistry for Nesp55

Initial characterisation of the Nesp55 primary antibody was required as it was a new bleed of antibody (obtained from Prof. R. Fischer-Colbrie, Innsbruck Medical University, Austria) that had not yet been characterised (unlike the β-gal antibody which was available commercially). Initial validation was carried out by completing immunohistochemistry staining on brain sections from WT, Nesp\textsuperscript{m/+} and Nesp\textsuperscript{+/p} adult mice (Figure 7.7). Staining revealed that the Nesp antibody successfully targeted the Nesp55 protein, showing cell specific staining in samples from both WT and Nesp\textsuperscript{+/p} mice (whereby maternal Nesp55 is present in the brain) in the hypothalamus, pons and midbrain regions, consistent with the discrete regions previously described (Plagge et al., 2005); and demonstrating an absence of staining in the brain tissue from Nesp\textsuperscript{m/+} (whereby maternal Nesp55 is absent).
Figure 7.7 Immunohistochemistry staining for Nesp in coronal sections of the adult mouse brain. As expected, the presence of discrete expression of Nesp was observed in the hypothalamus in both WT (a) and Nesp<sup>+/p</sup> (b) mice, but not Nesp<sup>m/+</sup> mice (c). Further examination of WT tissue showed distinct staining in different nuclei of the hypothalamus (d), specifically the dorsomedial hypothalamic nucleus (DM), arcuate hypothalamic nucleus (Arc), and the lateral hypothalamus (LH), and in the midbrain (e), the Edinger-Westphal nucleus (EWN) and in the pons (f), specifically the locus coeruleus (LC).
7.3.3 Immunofluorescence for expression of Nesp55 and Grb10

7.3.3.1 Initial characterisation of specific antibodies to be used

Characterisation of antibodies was carried out to ensure the specificity of the fluorescent staining. Control staining was carried out on Nesp55m/+ (where Nesp55 is not present in the brain) and a WT sibling, under the same experimental conditions. The Nesp55 antibody showed discrete staining in areas consistent with previous research in WT mouse brain (Plagge et al., 2005), and no discrete staining in Nesp55m/+ tissue (Figure 7.8). Furthermore antibodies for 5-HT and TH showed discrete staining in regions previously shown (Kolmac and Mitrofanis, 1999, Heisler et al., 2007), and no staining in negative controls (Figure 7.9a-b and d-e). Finally the β-gal antibody successfully targeted the reporter gene LacZ in Grb10+/p brain tissue, showing discrete staining in expected regions (Garfield et al., 2011) and no presence in WT brain tissue (Figure 7.9c and f).
Figure 7.8 Control immunofluorescence staining of Nesp55 in coronal sections of adult \textit{Nesp}^{m/+} and WT mice. As previous research (Plagge, et al. 2005) has shown the Nesp55 is mainly expressed in regions of the mid- and hindbrain, only brain sections from these regions were assessed (at level bregma= -0.82mm). In tissue from a \textit{Nesp}^{m/+} mouse, where Nesp55 is not present in the brain, there were no positive cells labelled with the antibody specific to Nesp55, (a-c). However, in WT brains, staining of Nesp55 was consistent with previous research (Plagge et al., 2005), showing discrete areas of dense reaction in the lateral hypothalamus (LH), Edinger-Westphal nucleus (EWn), and locus coeruleus (LC).
Figure 7.9 Control immunofluorescence staining of 5-HT, TH and β-gal in coronal sections of adult Grb10<sup>+/p</sup> mice. As previous research (Garfield et al., 2011) has shown the Grb10 is mainly expressed in regions of the mid- and hindbrain, only brain sections from these regions were assessed (at level bregma= -1.28mm). In the absence of primary antibodies, used as a control, there were no significant signs of reactivity for 5-HT (a), TH (b) and β-gal (c) in brain sections from Grb10<sup>+/p</sup> mice, as expected. However, in the presence of the primary antibodies, reactivity was observed for 5-HT in the hypothalamus (HYP) (d), TH in the zona Incerta (ZI) (e) and β-gal in the ventral tegmental area (VTA) (f).
7.3.3.2 Co-localisation of Nesp55 with Grb10 in Grb10+/− brain

To assess if Nesp55 is co-localised with Grb10, dual-labelled immunofluorescence was carried out in sections from Grb10+/− animals, where the reporter gene LacZ is expressed in place of Grb10. Therefore LacZ can be targeted using an antibody for β-gal, as a proxy for Grb10. Sections were therefore dual-labelled with antibodies to Nesp55 and β-gal, imaged at separate fluorescent wavelengths and finally the images merged (Figure 7.10). Inspection of the merged images revealed evidence for the co-localisation of Nesp55 and β-gal (Grb10) in the same cells in the LC, HYP and DRN.
**Figure 7.10 Dual-labelling immunofluorescence histochemistry of Nesp55 and Grb10 in coronal sections of an adult WT Grb10<sup>+/p</sup> mouse.** Sections were dual-labelled with antibodies against Nesp55 and β-gal, where the reporter gene LacZ is expressed in place of Grb10 in tissue from Grb10<sup>+/p</sup> mice, and can be used to identify Grb10-positive cells, images were viewed at different light intensities (568nm and 488nm for Nesp55 and b-gal, respectively), and were then merged to gauge cellular co-localisation of the two target proteins, depicted by white arrows in the merged figures. A number of cells showed evidence for co-localisation: within the locus coeruleus (LC) (a-c), the hypothalamus (HYP) (d-f) and the dorsal raphe nuclei (DRN) (g-i). LC and HYP images at x40 magnification, DRN at x20.
7.3.3.3 Co-localisation of Nesp55 with 5-HT and TH in WT brain

To assess if Nesp55 is co-localised with 5-HT and/or TH in cells of the mid- and hindbrain, dual-labelled immunofluorescence was carried out in sections from unmanipulated WT animals. Sections were therefore dual-labelled with antibodies to Nesp55 and 5-HT (Figure 7.11) or TH (Figure 7.12), imaged at separate fluorescent wavelengths and finally the pairs of images merged. Inspection of the merged images revealed evidence for the co-localisation of Nesp55 and 5-HT in the same cells, of the LC (Figure 7.11a-c), HYP (Figure 7.11d-f), but not in the EWn (Figure 7.11g-i). Dual-staining of Nesp55 and TH, showed no evidence for co-localisation of these two proteins in any of the regions investigated including the periaqueductal grey (PAG)(Figure 7.12a-c), HYP (Figure 7.12d-f) and the EWn (Figure 7.12g-i).
Figure 7.11 Dual-labelling immunofluorescence histochemistry of Nesp55 with 5-HT in coronal sections of an adult WT mouse. Sections were dual-labelled with antibodies against Nesp55 and 5-HT, images were viewed at different light intensities (568nm and 488nm for Nesp55 and 5-HT, respectively), and were then merged to gauge cellular co-localisation of the two target proteins, depicted by white arrows in the merged figures. A number of cells showed evidence for co-localisation: within the locus coeruleus (LC) (a-c), and the hypothalamus (HYP) (d-f), but not the edinger westphal nucleus (EWN) (g-i). LC and HYP images at x40 magnification, EWN at x20.
Figure 7.12 Dual-labelling immunofluorescence histochemistry of Nesp55 with TH in coronal sections of an adult WT mouse. Sections were dual-labelled with antibodies against Nesp55 and 5-HT, images were viewed at different light intensities (568nm and 488nm for Nesp55 and TH, respectively), and were then merged to gauge cellular co-localisation of the two target proteins. There was no evidence for co-localisation: within periaqueductal grey (PAG) (a-c), the hypothalamus (HYP) (d-f), or the Edinger Westphal nucleus (EWN) (g-i). All images were at x40 magnification.
7.4 Discussion

In order to begin to understand the neural roles of *Nesp* and *Grb10*, the expression profiles of these genes in the brain were examined. Real time-PCR (qPCR) analysis was used to examine the amount of expression of *Nesp* in the *Grb10*+/− mouse brain, as well as the converse of this, *Grb10* expression in *Nesp*+/− mouse brain. The results showed that the level of expression of *Nesp* is unaltered in *Grb10*+/− mice compared to WT mice, in both the hypothalamus and midbrain regions. Similarly the expression of *Grb10* was not significantly altered in the brain of *Nesp*+/− mice, in these regions also. Therefore, these data suggest that lack of either *Nesp* or *Grb10* does not affect expression (i.e. down- or up-regulation) of the other gene (i.e. *Grb10* and *Nesp*, respectively) in the brain regions analysed. However protein-protein interaction assays could be utilised to examine if Nesp55 and Grb10 are interacting biochemically or function separately.

Although it is known that *Nesp* and *Grb10* are expressed in the same brain regions (see Figure 7.1) (Plagge et al., 2005, Garfield et al., 2011), it is not known if Nesp55 and Grb10 are present in the same cells within these areas. Previous research examining the expression of *Nesp* in the brain showed that *Nesp* mRNA is not expressed widely in all neurons of the rat brain, nor is its distribution restricted to a single transmitter system or cell population (Bauer et al., 1999). Therefore, it is thought that Nesp55 may have functional significance for several neurotransmitter systems (Plagge et al., 2005). The main sites of *Nesp* expression are the noradrenergic and serotonergic systems. These are systems in which *Grb10* and *Nesp* have overlapping expression (Plagge et al., 2005, Garfield et al., 2011). Grb10 has previously been shown to be co-localised with serotonergic, cholinergic and dopaminergic neurons (Garfield et al., 2011), suggesting that Grb10 may too be important in the intracellular pathways of neurotransmitters that are involved in impulsivity and risk-taking behaviour; however it was unknown if Nesp55 co-localises with serotonergic or noradrenergic markers. In order to address these questions, dual-labelling immunofluorescence was carried out to discover if Nesp55 is expressed in the same cells as Grb10, and additionally if Nesp55 is co-localised with the neurotransmitter 5-HT and Tyrosine Hydroxylase (TH) a rate limiting enzyme for catecholamine synthesis.
In answering the first point, dual-labelled immunofluorescence staining was achieved using a previously characterised antibody for Nesp55 (Bauer et al., 1999, Plagge et al., 2005), and an antibody for β-gal as a substitute for Grb10 expression; importantly this has been validated in previous experiments (Garfield et al., 2011), and is therefore considered a valid proxy. Dual-labelled immunofluorescence revealed that Nesp55 and Grb10 were detectable in many of the same neurons of the different brain regions analysed (Figure 7.10); specifically showing co-localised expression in the LC, HYP, and DRN. The expression of these two proteins was not completely overlapping, whereby Nesp55 appeared to be expressed in parts of the cell where Grb10 was not expressed. However both Nesp55 and Grb10 appeared to be predominantly perinuclear in their expression, Nesp55 was particularly abundant in the perinuclear region, which is typical for this protein (Bauer et al., 1999). The discovery that these two imprinted genes are not completely segregated in the brain, and appear to be in the same cells has important implications for the behavioural effects that have been found in Grb10+/p and Nesp m/+ mice, both previously and presented in the current thesis. Specifically the finding that Grb10 and Nesp55 appear to oppositely affect measures of risk-taking/impulsive behaviour, such as novelty exploration and social dominance, as well as delay discounting behaviour (Chapter 4), and additionally are co-expressed in some cells would suggest that the Grb10 and Nesp55 proteins could influence these behaviours via the same neural systems, such as the noradrenergic or serotonergic systems. However future work using more in-depth molecular analysis would be required to confirm this hypothesis. Although this histological analysis provides evidence that Nesp55 and Grb10 may be influencing the same behaviours via the same neural systems, Grb10 is also expressed in other parts of the brain (Garfield et al., 2011). For example the expression of Grb10 in the SNC gives rise to the possibility that it may influence impulsive behaviour via a completely different system to Nesp55, namely the dopaminergic system. Finally, the dense presence of Nesp55 and Grb10 in the hypothalamus opens the possibility of these two genes influencing numerous other behaviours, including functions such as the stress response, feeding behaviour and sexual function (Bauer et al., 1999).

The dual-labelled immunofluorescence staining also revealed that Nesp55 was detectable in serotonergic neurons; showing distinct punctate areas of co-localisation
(yellow) within parts, but not all, of the cell body. The 5-HT antibody gave a widespread staining throughout the cells in the LC and HYP, which was similar in appearance to the distribution of the Nesp55 protein. Merged images indicated significant but not total co-localisation of 5-HT and Nesp55. Specifically Nesp55 distribution was perinuclear, and confined to the stoma of the cell; whilst 5-HT was expressed in both the stoma and the processes of the cell (Figure 7.11e). Furthermore Nesp55 appeared to be present in many more cells in the hypothalamus than 5-HT, showing that 5-HT and Nesp55 overlap was not exclusive. No such co-localisation was found in the EWn, whereby there was distinct staining of Nesp55, as previously found (Plagge et al., 2005), but no presence of 5-HT, as was expected. The staining of Nesp55 in the EWn (Figure 7.11g) shows the presence of this protein in the processes of the cell, indicating vestibular transport of Nesp55 to this site. These data imply that although Nesp55 is expressed in some serotonergic neurones, the expression is not universal or exclusive to this neurotransmitter system. Nevertheless, these data provide further evidence for the suggestion that Nesp55 exerts an influence on impulsive/risk-taking behaviour via the serotonergic system.

Previous research has shown that the expression of Nesp overlaps nearly completely with brain regions of the noradrenergic, adrenergic and serotonergic systems (Bauer et al., 1999). However the immunofluorescence results presented here showed that Nesp55 was not in the same cells as TH, showing distinct expression in separate cells that did not overlap in the PAG, HYP or EWn. TH is a marker for dopamine, noradrenaline, and adrenaline containing (catecholamine) neurons and endocrine cells (Weihe et al., 2006). It is therefore somewhat surprising that Nesp does not appear to be expressed in neurons of this kind. It would be of interest to assess co-localisation of Nesp55 and NA specifically, in order to establish if Nesp55 is present in noradrenergic neurons, despite not being present in TH containing neurons.

The data presented here provide important preliminary findings, which contribute to current understanding of the molecular functioning, and interactions of Nesp55 and Grb10 in the brain. It would therefore be of interest to analyse Nesp55 and Grb10 protein levels, instead of mRNA, in order to better understand if they are altered in the KO mouse of the opposite gene. The immunofluorescence data presented here provides support for the idea of co-localisation of Grb10 and Nesp55, as well as Nesp55 and 5-HT; however the limited
resolution capabilities of the fluorescence microscopy techniques mean that they should be treated cautiously. Verification with more advanced microscopy techniques such as confocal microscopy or super resolution microscopy, are necessary to confirm these data. If these proteins are confirmed to be in the same cells, it would be of interest to carry out a proximity-ligation assay (PLA), which allows the fluorescent visualisation of protein proximity in situ (Weibrecht et al., 2010). This technique is highly selective and therefore would accurately confirm if Nesp55 is localised in the same cells as 5-HT and Grb10, and whether it is in close enough proximity to suggest an interaction. Protein-protein interaction could then be further investigated by co-immunoprecipitation or western blotting. However, despite these caveats, based on the preliminary data here it can be concluded that *Nesp* is expressed neuronally, and may be contributing to the same neural circuitry as 5-HT. Furthermore the finding that Nesp55 and Grb10 appear to be in the same cells provides the first piece of evidence that these imprinted genes could influence adult behaviour via the same neural systems. Moreover the co-localisation with 5-HT and lack of co-localisation with TH, points toward this influence being via the serotonergic system.

### 7.4.1 Summary of key results from Chapter 7

- qPCR data revealed no difference in amount of expression of *Nesp* and *Grb10* in *Grb10*+/− and *Nesp*+/− mice, respectively, compared to WT littermates.
- Dual-labelled immunofluorescence revealed that Nesp55 appeared to be co-localised in the same cells as Grb10.
- Dual-labelled immunofluorescence revealed that Nesp55 appeared to be expressed in serotonergic cells, but not in the same cells as tyrosine hydroxylase.
Chapter 8 – General Discussion

8.1 Overview

The overall aim of this thesis was to compare mice null for brain expression of Nesp55 and Grb10 to test whether these two oppositely imprinted genes influence the same behaviours, specifically impulsivity/risk-taking. The rationale for this was that these genes show overlapping expression in discrete brain regions related to these behaviours and therefore may be influencing the same functions, similar to the actions of other co-expressed imprinted genes in other physiological systems (Constancia et al., 2004). This hypothesis was given further credence by findings presented in Chapter 7, which suggests that in some cells within those overlapping brain regions, Nesp55 and Grb10 are also co-expressed (discussed further below).

The behaviours assessed in Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice were chosen on the basis of the expression patterns of Nesp and Grb10 in the brain and, particularly on the basis of previous behavioural analyses. Behaviourally, it has been shown previously that Nesp\textsuperscript{m/+} animals demonstrate a reluctance to explore novel environments (Plagge et al. 2005), whereas Grb10\textsuperscript{+/p} mice exhibit a number of phenotypes that suggest a role for this gene in mediating social dominance (Garfield et al., 2011). Although these behaviours seem unrelated, it has been proposed that they can both be viewed as indicative of altered risk-taking and/or impulsivity generally (Isles et al., 2006, Davies et al., 2008). However, further and more explicit tests were required to ascertain if and how Nesp55 and Grb10 may influence these functions. Nevertheless, investigation into this aspect of behaviour is particularly interesting due to its recognition as a clinical symptom of various psychiatric disorders, including schizophrenia, attention deficit hyperactivity disorder (ADHD) and obsessive compulsive disorder (OCD). This paired with the fact that some imprinted genes have already been associated with the incidence of neuropsychiatric illness (McNamara and Isles, 2013), leads to important possibilities about Nesp55 and Grb10 having a potential role in human behavioural disorders.
The specific experimental aims of this thesis were to:

- Assess $Grb10^{+/p}$ in the behavioural assays previously found to be altered in $Nesp^{m/+}$ mice, including the locomotor activity, novelty place preference and delayed reinforcement tasks.
- Develop a novel, more ethobiologically relevant test of risk-taking behaviour, the predator odour risk taking (PORT) task, and use this task to examine if risk-taking is altered in $Nesp^{m/+}$ and $Grb10^{+/p}$ mice.
- Analyse the expression profiles of $Nesp$ and $Grb10$, including examining if they are co-localised within the same cells, whether loss of expression of one gene has consequences for the expression of the other; and finally, if $Nesp$ is co-localised with neurotransmitters central to the control of impulsivity/risk-taking behaviour.

### 8.2 Main findings

A summary of the results of the behavioural assays can be found in Table 8.1. Briefly, previous experiments assessing the effect $Nesp$ has on behaviour, found that $Nesp^{m/+}$ mice had an increased locomotor activity on the first day of 3 day LMA testing. Furthermore $Nesp^{m/+}$ mice made an significantly increased number of entries into, but spent less time in, a novel environment in the novelty place preference (NPP) task, behaviours which were not driven by altered anxiety (Plagge et al., 2005). Taken together this showed that $Nesp^{m/+}$ mice had increased excitement towards, but are less willing to explore, a novel environment. This pattern of behaviour has been suggested to be indicative of altered risk-taking (Plagge et al. 2005., Isles et al. 2006). In the same experiments, presented in this thesis, $Grb10^{+/p}$ mice showed no difference to WT littermates in behaviour, completing an equivalent amount of activity in the LMA, and making a similar number of entries into, and spending a similar amount of time in, the novel environment of the NPP task (Chapter 3). This suggests that $Grb10$ does not influence novelty exploration, in the way that $Nesp$ has been shown to.
Table 8.1 Summary of behavioural findings of Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice from previous and current research.

Results from the behavioural assays carried out in the two transgenic lines of mice indicate that both Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice have altered behaviour in the delay-discounting task.

* Represents previous research, all other results represent those presented in this thesis, except ** which represents data not presented in this thesis.

Previous research also assessed behaviour in a delay-discounting task, and found that Nesp\textsuperscript{m/+} mice were significantly quicker to discount the large delayed reward in favour of the small immediate reward relative to their WT littermates (Isles et al., unpublished data). As presented in the current thesis, Grb10\textsuperscript{+/p} mice showed the opposite behaviour in the same task of delay-discounting behaviour; whereby Grb10\textsuperscript{+/p} mice were significantly more willing to wait for the large reward relative to WT littermates (Chapter 4). Furthermore, the opposite behaviour of Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice in the delay-discounting task was not due to an effect of motivation. This was demonstrated by results from the progressive ratio task (PRT), which indicated that mice lacking Grb10 expression showed equivalent levels of motivation to work (nose poke) for a reward. Although not presented in the current thesis, Nesp\textsuperscript{m/+} mice were also assessed in the PR task, and also demonstrated an equivalent level of motivation to work for the reward. Thus, Nesp and Grb10 appear to have opposing effects on delay-discounting behaviour specifically.

In order to further understand the specific behaviours that Nesp and Grb10 affect, it was of interest to examine Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice in a further explicit test of risk-taking behaviour with greater ethobiological relevance. I successfully developed a novel risk-taking task, the Predator Odour risk-taking (PORT) task (Dent et al., 2013), which examined the propensity to traverse a chamber to retrieve a reward whilst under the potential threat of a predator (Chapter 5). The PORT task adds to the growing number of tasks which approach...
risk-taking from a ‘real-world’ perspective, and uses an ethobiologically relevant environment with high levels of control (Olsson et al., 2003, Choi and Kim, 2010, Franks et al., 2012). This task was first validated in WT mice of the same background strain as Nesp\(m^+/\) and Grb10\(r/p\) mice (Chapter 5). Using this task it was demonstrated that mice will readily traverse a central chamber containing their own odour to collect a reward; however when the odour of a predator is present, all mice systematically changed their behaviour and became more reluctant to traverse the central chamber to collect the reward. Therefore, the latency with which they collected the reward gauged the level of risk-taking. In this task it was demonstrated that both Nesp\(m^+/\) and Grb10\(r/p\) mice showed behaviour indicative of normal levels of risk-taking (Chapter 6), specifically showing equivalent latencies to collect the reward to their own WT littermates.

Finally, in an attempt to understand the neurological correlates of the behaviour presented in this thesis, an investigation into expression patterns of Nesp and Grb10 was carried out (Chapter 7), to extend the previous literature (Plagge et al., 2005, Garfield et al., 2011). First, qPCR was used to investigate if knock-down of each gene affected expression of the other. Results showed that there were no differences in the amount of Nesp55 expression in either the midbrain or hypothalamus of Grb10\(r/p\) and WT mice, or Grb10 expression in the same brain regions of Nesp\(m^+/\) and WT littermate mice. Therefore, altering the expression of each gene did not affect the expression of the other. Dual-labelled immunofluorescence histochemistry was used to identify if Nesp55 and Grb10 are expressed in the same cells in regions of the brain in which they have overlapping expression, such as the locus coeruleus (LC), hypothalamus (HYP) and dorsal raphe nucleus (DRN). The results provided evidence for a high-level of co-localisation between Nesp55 and Grb10 in each of these areas. This interesting result may be indicative of an interaction between these two proteins, and thus may underlie the mechanism for antagonistic behaviour found in the Nesp\(m^+/\) and Grb10\(r/p\) mice. Moreover, both Grb10, in previous research (Garfield et al., 2011), and Nesp55 in the current research, were found to be present in serotonergic neurons. Serotonin is one of the major neurotransmitters that regulate impulsivity and risk-taking behaviour (Winstanley et al., 2003, Dalley and Roiser, 2012). Current research showed that Nesp55 was not found to be co-localised in the same cells as tyrosine hydroxylase (TH), which is a marker for dopamine, noradrenaline and
adrenaline containing cells. Therefore based on the expression analysis data presented in the current thesis as well as previous research (Garfield et al., 2011) it could be suggested that it may be through the serotonergic system that Nesp55 and Grb10 act in order to influence delay-discounting behaviour. However the involvement of the dopamine and noradrenaline systems has not been ruled out, and would be an important aspect for future investigation in order to determine the mechanisms through which Nesp55 and Grb10 affect behaviour.

8.3 Discussion of findings

The functional role of genomic imprinting remains an interesting and largely unresolved phenomenon. Although our understanding of imprinted gene regulation and physiological relevance has developed considerably, the reason for the existence of this subset of genes remains conjecture until their function has been completely characterised throughout the body and the brain. Recent advances have suggested that there may be significantly more brain-expressed imprinted genes in the genome than first thought (Gregg et al., 2010); this coupled with their growing association with psychiatric illness (Ingason et al., 2011b, Rees et al., 2013) and important role in development leave intriguing possibilities about what exactly imprinted genes may do, and why they have evolved.

The functions that have already been shown to be influenced by imprinted genes suggest they have important effects on a number of aspects of adult behaviour (Plagge et al. 2005., Garfield et al., 2011). The aim of this thesis was to investigate the brain role of the imprinted genes Nesp and paternal Grb10, as these two genes provide an example of where neural and in utero growth functions dissociate. Moreover, these two oppositely imprinted genes show discrete but highly overlapping patterns of expression in the brain, reflecting the current suggested “hot-spots” of imprinting in the mouse brain, namely the HYP, Edinger-Westphal nucleus (EWN) and monoaminergic mid-brain regions including the LC, DRN and ventral tegmental area (VTA) (Gregg et al., 2010). Advances in targeted mutagenesis have provided an excellent opportunity to investigate these genes, and specifically how they might be controlling behaviour. Research employing the use of animal
models where particular imprinted genes can be targeted and deleted, have begun to uncover a fascinating role for imprinted genes in mediating adult behaviour (Isles and Wilkinson, 2000, Davies et al., 2008).

Prior to this research no direct antagonistic effect on behaviour had been observed for any imprinted genes, although previous studies of Nesp and paternal Grb10 knock-out models raised some interesting possibilities (Plagge et al. 2005, Garfield et al. 2011). The suggested involvement of Nesp and Grb10 in the control of impulsive/ risk-taking behaviour, may be significant for clinical populations. Pathological levels of impulsivity and risk-taking is a significant symptom in serious mental illnesses, such as schizophrenia and bipolar (Reddy et al., 2014), as well as gambling and drug addiction (Verdejo-García et al., 2008). The previously identified behavioural phenotypes seen in both Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice could be seen under the umbrella of risk-related behaviour (Plagge et al. 2005,. Garfield et al. 2011). In a naturalistic environment, novelty exploration, as is altered in Nesp\textsuperscript{m/+} mice, would be considered risky behaviour due to an innate fear of predation (Cryan and Holmes, 2005, Choi and Kim, 2010). Similarly, social dominance behaviour, as is altered in Grb10\textsuperscript{+/p} mice, may also be correlated with the propensity to take risks (Davis et al., 2009). Social dominance is achieved through a series of aggressive encounters, where access to food, water and reproductive partners are the incentive. Thus, social dominance is indicative of taking the risk of engaging in an aggressive encounter, where there is a possibility of harm or loss of status (Sapolsky, 2005). It has now been demonstrated that as well as this existing evidence, Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice have opposite delay discounting behaviour, whereby Nesp\textsuperscript{m/+} mice have a significant preference for the small reward, whilst Grb10\textsuperscript{+/p} mice have a significant preference for the large reward.

Delay discounting has been predominantly used to measure choice impulsivity in humans (Reynolds, 2006) and laboratory animals (Winstanley et al., 2003). However there is evidence to suggest that delay discounting could also be interpreted as risk-taking behaviour in animals, due to the understanding that animals view temporally distant rewards as more risky (Hayden and Platt, 2007, Kalenscher, 2007). Based on this interpretation it could therefore be concluded that Nesp\textsuperscript{m/+} mice are less risk-taking, due to preference for the ‘safe’ temporally immediate reward, and Grb10\textsuperscript{+/p} mice are more risk-
taking, due to preference for the ‘risky’ temporally distant reward. This interpretation agrees with previous findings that Nesp\textsuperscript{m/+} mice were risk-averse in the NPP, LMA and open field tests of novelty exploration. Based on this interpretation it could be suggested that Nesp and Grb10 have antagonistic roles in mediating risk-taking behaviour. However in an explicit test of risk-taking behaviour, the PORT task, it was found that both Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice exhibited normal behaviour. The finding that neither Nesp\textsuperscript{m/+} nor Grb10\textsuperscript{+/p} mice differed from WT littermate mice in risk-taking behaviour in the PORT task, was somewhat surprising, due to the altered delay-discounting behaviour, as well as previous alterations in novelty exploration and social dominance (in Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice respectively). However the finding that there was no evidence from the PORT task to suggest altered risk-taking behaviour in Grb10\textsuperscript{+/p} mice may also indicate that the altered delay-discounting phenotype measured here is not risk-taking behaviour, but is indeed altered impulsivity, and specifically impulsive choice. Involvement of these two genes in this facet of behaviour is not only interesting, but clinically relevant, as abnormalities in impulsive choice behaviour and specifically delay discounting behaviour are associated with psychiatric disorders such as pathological gambling and drug addiction (Petry, 2001, Alessi and Petry, 2003).

In order to confirm that the effect found in delay discounting behaviour is specific to impulsive choice, the decisional aspect of impulsivity, it would be interesting to examine Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice in an assay of impulsive action, the motoric aspect of impulsivity. Since the completion of the experiments presented in this thesis, Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice have subsequently been examined in the stop-signal reaction time task (SSRTT) (Lewis and Dent, 2013 unpublished data). The SSRTT, a test of ‘impulsive action’ or ‘motor impulsivity’ (Winstanley et al., 2010) evaluates response inhibition through measuring the ability to stop or cancel a motor action that has already been initiated, in response to a ‘stop-signal’. This task has been used widely in the clinical setting and with rats, and has recently been successfully translated for use with mice (Humby et al., 2013). Deficits in the ability to stop or ‘inhibit’ a pre-potent response have been associated with pathological conditions such as ADHD (Rubia et al., 2007), are sensitive to dopaminergic, serotonergic and noradrenergic manipulations (Humby et al., 2013), and furthermore, have been shown to have a dissociation from impulsive choice (Eagle et al., 2008, Robinson et al., 2008). During the task
for mice, following an initial response, subjects learn to make a second (rapid) nose poke response when cued to do so by a ‘go’ signal, however on some trials a ‘no-go’ (stop) signal is presented after the presentation of a ‘go’ signal. By varying the position of the stop-signal within the go response the ability to inhibit a response once it has already been initiated can be evaluated, where the main measure of impulsivity is considered at the point where the go and stop responses compete (50% correct stopping) (Winstanley et al., 2006). Using this task, it was demonstrated that $Nesp^{m/+}$ and $Grb10^{+/p}$ mice both successfully learnt the task, and showed no difference in the ability to withhold a pre-potent response, demonstrated by comparative stop-signal reaction times (calculated at ~50% correct stopping) (Figure 8.1). $Nesp^{m/+}$ and $Grb10^{+/p}$ mice do not have altered response inhibition in this task, indicating that $Nesp$ and $Grb10$ do not exert an influence on impulsive action. This suggests that the function of these two imprinted genes on impulsivity, is predominantly restricted to only impulsive choice behaviour.

**Figure 8.1** $Nesp^{m/+}$ and $Grb10^{+/p}$ mice performance in the stop-signal reaction time task (SSRTT). Equivalent SSRTs (calculated at 50% correct stopping position) were observed in both $Nesp^{m/+}$ (Figure 8.1a; one-way ANOVA, main effect of GENOTYPE, $F_{1,28}=0.12, P=0.74$) and $Grb10^{+/p}$ cohorts (Figure 8.1b; one-way ANOVA, main effect of DELAY, $F_{1,21}=0.07, P=0.79$). $Nesp$ cohort: WT N=12 $Nesp^{m/+}$ N=17, $Grb10$ cohort: WT N=13, $Grb10^{+/p}$ N=9.

$Nesp$ and $Grb10$ have been found to have overlapping expression in areas that have been shown to influence impulsive behaviours, such as the HYP, EWn, DRN, and LC (Plagge et al., 2005, Garfield et al., 2011). As such the underlying neural basis of the antagonistic
delay discounting behaviour found in *Nesp*\(^{m/+}\) and *Grb10*\(^{+/p}\) brain tissue was investigated and found that, like *Grb10* (Garfield et al., 2011), Nesp55 was co-localised in the same cells as serotonin in both the HYP and the LC, however not in the EWn (Chapter 7). Furthermore, the Nesp55 and Grb10 proteins were also found to be co-localised within the same cells, in the HYP, LC and DRN, although qPCR investigation did not find evidence for altered expression of each gene in the absence of the other. The co-localisation evidence points towards the possibility that Nesp55 and Grb10 may exert their influence on the same or similar neurological pathways, and specifically via the serotonergic system. However, despite the significant overlap in expression, the additional expression of *Grb10* in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNC) also allows the possibility that Nesp55 and Grb10 could be influencing impulsive behaviour via entirely separate systems. For instance *Grb10* may influence this behaviour via the dopaminergic system.

Due to knocking out *Nesp* and *Grb10* on two separate mouse lines a direct comparison cannot be drawn between the two groups of KO subjects; *Nesp*\(^{m/+}\) and *Grb10*\(^{+/p}\) subjects can only be compared to their respective WT littermates. However in future research it would be interesting to generate a double knock-out of both *Nesp* and *Grb10* in a single mouse line, in order to assess the impact of this upon impulsive and risk-taking behaviours. This would allow an insight into whether *Nesp* and *Grb10* are interacting and the systems upon which they are acting. If an effect on behaviour was found in double KO subjects the direction of effect would determine whether the action of one gene is upstream of the other. Alternatively if the behaviour was the same as WT subjects, this would be indicative of *Nesp* and *Grb10* acting in the same pathway but cancelling each other out, or acting on different pathways that influence the same function. For example this may confirm the premise that *Nesp* acts via the serotonergic system and *Grb10* acts via the dopaminergic system.

The concept that the oppositely imprinted genes (maternal) *Nesp* and (paternal) *Grb10* have antagonistic effects on impulsive behaviour is not the first demonstration of this kind. In fact it adds to a growing number of examples of imprinted genes in plants and animals that seem to have antagonistic roles (Moore and Haig, 1991, Kinoshita et al., 2008). This concept is explained by the conflict (or kinship) theory of imprinted genes, which is
based on the premise that parental alleles act to promote their own interests, resulting in antagonistic action between maternal and paternal alleles (Haig, 2000). It is proposed that paternally expressed genes maximise the father’s reproductive success through promoting embryonic and early post-natal growth by extracting nutritional resources from mother. This theory is most prominent in the case of the paternally expressed imprinted gene *Igf2* which promotes foetal growth, and the maternally expressed imprinted gene *Igf2r* which restricts foetal growth (Haig and Graham, 1991). The results presented in this thesis are in agreement with the conflict theory of imprinted genes (Haig, 2000); whereby *Nesp* and *Grb10* which are oppositely imprinted genes show antagonistic effects on impulsive behaviour. However further investigation into the molecular and network mechanisms of *Nesp*55 and *Grb10* and their possible interactions is required to determine if the opposite behaviour found in the transgenic mice is a result of antagonism at a molecular level. Whilst the behavioural effects of these two genes are in agreement with theories, and increasing evidence suggesting that oppositely imprinted genes can be in conflict when governing functions (Moore and Haig, 1991, Haig, 2000), what functions *Nesp* and *Grb10* are in conflict about and the underlying mechanism of this relationship remains unclear.

### 8.4 Future behavioural directions

#### 8.4.1 Previous behaviour

In future research, in order to investigate the specificity of the influence on *Nesp* and *Grb10* further, it would be of interest to test *Nesp*\textsuperscript{m/+} and *Grb10*\textsuperscript{+/p} on further tests of impulsive choice and risk-taking behaviours. The different findings from the battery of behavioural tests presented in this thesis may be reflective of the multi-faceted nature of decision-making behaviour. Human studies have found that performance in the Iowa gambling task and the Balloon Analogue Risk Task (BART) correlate with “real-world” risky behaviour in healthy controls and clinical populations such a drug-abusers, alcoholics and problem gamblers (Schonberg et al., 2011). This is supported by work with rats showing that individual behaviour in the operant Risky Decision Making (RDM) task correlates with performance in a probabilistic discounting task (Simon et al., 2009). In contrast this same
study found no correlation between behaviour in the RDM task and a delay discounting task (Simon et al., 2009). Therefore it would be of interest to test Nesp<sup>+</sup> and Grb10<sup>+</sup> mice in operant tests of risk-taking/impulsive choice, such as rodent analogues of the probabilistic discounting task, Iowa gambling task, and the BART. With a possible hypothesis that Nesp<sup>+</sup> mice may exhibit decreased risk-taking behaviour, whilst Grb10<sup>+</sup> mice may exhibit increased risk-taking behaviour. It would also be interesting to correlate the behaviour measured in tasks such as these and behaviour in the PORT task; in order to better understand if the PORT task is taxing the same or separate risk-taking processes as existing tests of risk-taking. Furthermore this would determine if Nesp and Grb10 affect impulsive choice generally or specifically affect delay-discounting behaviour. Based on previous research (Simon et al., 2009) and the research presented here, it is hypothesized that behaviour in the PORT task would correlated with existing tests of risk-taking/impulsive choice behaviour, such as those mentioned above.

The principle limitation of the PORT task is its vulnerability to habituation, whereby extended repeated exposure to a predator odour would allow subjects to learn that there is no actual threat; therefore the task is not amenable to repeated-measures experimental designs. This limited our ability to carry out pharmacological experimentation, however future use of the PORT task with much larger cohorts of mice would prohibit a within-subjects design to further examine risk-taking behaviour in mice. Manipulations of dopamine and serotonin in animal studies have shown a profound effect on behaviour in risk-based decision making tasks (Zeeb et al., 2009). For instance, dopamine functioning in the brain, particularly D<sub>2</sub>-like receptors, are known to have profound effects on risk-taking behaviour (Norbury et al., 2013). Previous research with mice demonstrated that systemic activation of D<sub>2</sub>-like receptors attenuated risk-taking behaviour in the RDM task (Simon et al., 2011a). Therefore the use of dopamine agonists/antagonists may elucidate potential differences in the risk-taking behaviour of Nesp<sup>+</sup> and Grb10<sup>+</sup> mice. Evidence presented in this thesis that both Nesp55 and Grb10 are found in serotonergic neurons, suggests that the use of serotonin agonists/antagonists may also be a valuable manipulation to elucidate potential effects of Nesp and Grb10 on this aspect of behaviour. The importance of serotonin in risk-sensitive decision making has been shown previously, whereby administration of 5-HT receptor agonist impaired the ability of rats to perform a rodent
gambling task (Zeeb et al., 2009), furthermore reducing brain serotonin synthesis in macaques decreased preference for the safe option in a gambling task (Long et al., 2009).

Pathological impulsivity is a clinical symptom of numerous psychiatric disorders such as schizophrenia, where cognitive and memory-related difficulties are also highly prevalent (Heerey et al., 2007). Moreover it is thought that deficits in cognition and memory contribute to the impulsive decision making process (Heerey et al., 2007). Based on this concept it may also be valuable to assess if Nesp and Grb10 contribute to cognitive and memory functions also, such as attention and learning. Although ability to learn tasks (such as DRT, PR, SSRTT) was highly comparable between WT and mutant subjects for both the Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} lines, it would be of interest to examine these functions in explicit tests of attention, learning and memory in Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice, such as 5-choice, reversal learning and set-shifting paradigms (Bissonette and Powell, 2012). This may elucidate a further system through which Nesp and Grb10 influence impulsive behaviour, and may even suggest that deficits in cognition and learning may be driving the impulsive phenotype found in Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice.

### 8.4.2 Expression led behaviours

The expression of Nesp and Grb10 in the hypothalamus (Plagge et al., 2005, Garfield et al., 2011), and indeed the discovery of these transcripts in the same hypothalamic cells (see Figure 7.10) suggests a role for Nesp and Grb10 in mediating behaviours beyond those examined here. This thesis has principally focused on decision-making behaviours, however it is known that the hypothalamus also regulates motivation and goal-directed behaviours, such as feeding, drinking, aggression and sexual-behaviours (Sternson, 2013). Therefore it would be of interest to examine Nesp\textsuperscript{m/+} and Grb10\textsuperscript{+/p} mice in assays that examine behaviours such as food/drink seeking and consumption behaviours, such as lick-cluster analysis, or high-fat diet consumption, in order to determine if Nesp and Grb10 affect feeding and appetite. Furthermore, assays such as the resident-intruder paradigm could be used to examine social aggression (Koolhaas et al., 2013). Moreover, the previous finding that Grb10 influences social dominance in the tube-test (Garfield et al., 2011) may suggest a hypothalamic influence on behaviour. Social dominance in animals is a sexual/aggressive
behaviour essential for species propagation, therefore it is likely to be mediated by the hypothalamus (Sternson, 2013) and neurotransmitter projections to the forebrain (Wang et al., 2012). Therefore it would be of interest to examine Nesp<sup>m/+</sup> mice in the tube test also, to assess if Nesp<sup>m/+</sup> mice show altered social dominance. Further assays of social dominance such as the partition test (Kudryavtseva, 1994) and urine marking (Drickamer, 2001) could also be used to corroborate the social dominance phenotype found previously in Grb10<sup>+/p</sup> mice (Garfield et al., 2011), and to determine if Nesp has any influence on this behaviour also. Based on the findings in this thesis it would be hypothesized that Nesp<sup>m/+</sup> mice would have reduced social dominance, due to the finding that Grb10<sup>+/p</sup> mice have increased social dominance (Garfield et al., 2011), and Nesp and Grb10 were found to have conflicting effects on delay discounting behaviour. This assessment would allow us to better understand through which neuroanatomical regions Nesp and Grb10 are exerting an influence.

In addition to heightened social dominance in the tube test found in Grb10<sup>+/p</sup> mice, results of the tube test correlated with levels of ‘social barbering’, thought to be another measure of social dominance. Whereby the dominant mouse in a cage barbers the whiskers of cage-mates, known as the ‘Dalila effect’ (Sarna et al., 2000). However it has been argued that the social barbering observed in mice without expression of Grb10, is not indicative of social dominance/risk-taking, but is instead a type of pathological stereotypy, akin to the impulse control disorder Trichatillamania in humans (Garner et al., 2004). Social barbering could therefore be interpreted as impulsive behaviour and not risk-taking behaviour. Based on this hypothesis it could therefore be suggested that Grb10 exerts an influence on impulsive behaviour, evidenced by increased social barbering, a phenomenon that is not observed in Nesp<sup>m/+</sup> mice. This argument provides further evidence that the behavioural phenotype measured in Grb10<sup>+/p</sup> mice is that of impulsivity, not risk-taking behaviour. Furthermore expression of Grb10 in the VTA and SNC, areas where Nesp is not expressed, suggest that this behaviour may also occur via these areas, and specifically through the dopaminergic system.
8.5 Summary

In summary, the data presented in this thesis show that *Nesp* and *Grb10* both contribute to risk-taking and/or impulsive choice behaviour thus providing a novel mode of action for genomic imprinting in the brain. Results from expression analysis revealed the presence of *Nesp* and *Grb10* in the same cells of the LC, HYP and DRN, suggesting that these two imprinted genes may be influencing this behaviour through the same or similar signalling pathways. Previous evidence, as well as evidence presented in this thesis, point towards *Nesp* and *Grb10* mediating risk-taking/impulsive choice via serotonergic and possibly noradrenergic cells, in regions in which they have overlapping expression, such as the HYP, DRN and LC. The exact neural bases of imprinted gene action on delay discounting behaviour remain to be established. Nevertheless, this is the first evidence of an antagonistic action of maternally and paternally expressed imprinted genes on a brain function. Therefore this work indicates that paternal and maternal genomes can contribute differentially and perhaps antagonistically, not only on facets of impulsivity in the normal range, but also to pathological conditions such as gambling and drug addiction.
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