Foetal Programming of Brain Function and Behaviour:
A Behavioural and Molecular Characterisation of a Murine Placental Imprinted Gene Deletion Model

by

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Abbreviations

1-CSRTT	1-Choice Serial Reaction Time Task
5-CSRTT	5-Choice Serial Reaction Time Task
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine (serotonin)
ACTH	Adrenocorticotrophin hormones
ADHD	Attentional-deficit hyperactivity disorder
ANOVA	Analysis of variance
AVP	Arginine vasopressin
BWS	Beckwith-Wiedemann syndrome
BZ	Benzodiazepine
cDNA	Complimentary deoxyribonucleic acid
CNS	Central nervous system
COMT	Catechol *O*-methyl transferase
CPT	Continuous performance test
CRH	Corticotropin releasing hormone
D1	Dopamine receptor 1
D2	Dopamine receptor 2
DAT	Dopamine transporter
df	Degrees of freedom
DNA	Deoxyribonucleic acid
DOI	(±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
DOPAC	3,4–dihydroxy-phenylacetic acid
DSM-IV-TR	Diagnostic and statistical manual of mental disorders-IV text revision
E	Embryonic
EDTA	Ethylenediaminetetraacetic acid
EPM	Elevated plus maze
ES	Embryonic stem
FEP	Free exploratory paradigm

GABA	γ-aminobutyric acid
GANC	Gancyclovir
HSV	Herpes simplex virus
HPA	Hypothalamic-pituitary-adrenal
IUGR	Intrauterine growth restriction
Igf1	Insulin-like growth factor 1 gene (mouse gene)
Igf2	Insulin-like growth factor 2 gene (mouse gene)
IGF2	Insulin-like growth factor 2 gene (human gene)
Igf1r	Insulin-like growth factor 1 receptor gene (mouse gene)
Igf2r	Insulin-like growth factor 2 receptor gene (mouse gene)
IGF-I	Insulin-like growth factor 1 (protein)
IGF-II	Insulin-like growth factor 2 (protein)
IGF-IR	Insulin-like growth factor 1 receptor (protein)
IGF-IIR	Insulin-like growth factor 2 receptor (protein)
IGFBP	Insulin-like growth factor binding (protein)
INS	Insulin (human gene)
ITI	Inter-trial interval
kb	Kilobase
KO	Knockout
LBW	Low birth weight
LH	Limited hold
LMA	Locomotor activity
LTP	Long-term potentiation
M (m)	Mean
MagLat	Magazine latency
mRNA	Messenger ribonucleic acid
NACWO	Named animal care welfare officer
NMDA	N-methyl-D-aspartate
NTC	Non-template control
OF	Open field
PCR	Polymerase chain reaction
PIL	Personal licence

PPI	Prepulse inhibition
PPL	Project licence
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Reaction time
SAP	Stretch attend posture
SD	Stimulus duration
SEM	Standard error of measurement
SGA	Small for gestational age
SSD	Stop signal delay
SSRTT	Stop-signal serial reaction time task
TH	Tyrosine hydroxylaze
tk	Thymidine kinase
Tris	Tris(hydroxymethyl)aminomethane
WAIS-R	Wechsler Adult Intelligence Scale-Revised
WT	Wild-type

Summary

Foetal programming describes the process whereby exposure to environmental stimuli or insults during early development can lead to enduring change in structure and function in later life. This concept has found much support in terms of altered metabolic and cardiovascular function from a range of epidemiological and animal studies, but more recent work is highlighting the role that poor foetal nutrition may have on the development of behavioural and psychiatric problems. The biological mechanisms underlying nutritional programming remain uncertain, however compromised placental deficiency and/or foetal endocrine systems (particularly the IGF axis), have been considered to be of paramount importance at the nexus of this foetal-environmental interplay.

In the present study, two knockout mouse models of the imprinted *Igf2* gene were used to investigate the effects of placental deficiency and foetal growth restriction on brain development and behaviour in later life. Total deletion of the *Igf2* gene (*Igf2*-Null KO) leads to complete ablation of *Igf2* expression from all foetal and placental tissue, severe growth deficiency of both the foetus and placenta, and a mouse that is born small (50% of normal size) and remains small for life. However, the *Igf2*-P0 knockout (Igf2-P0 KO) model, where *Igf2* expression is only suppressed in the placenta (deletion of the P0 promoter) results in moderate foetal and placental growth retardation and an adult of equivalent size of normal. Intrauterine growth restricted mice from both *Igf2* knockout models were tested on a wide range of behavioural batteries, and performance compared with that of wild-type littermates. Since epidemiological studies of humans have commonly demonstrated linkage between nutritional compromise in early life, and ADHD symptoms and anxiety disorders in later life, behavioural tasks sensitive to these behavioural phenotypes were selected for the purpose of present experiments. *Igf2*-P0 KO mice displayed heightened levels of stress and anxiety, as indicated by greater startle responses and a marked increase in avoidance behaviour on several conflict-based tests of anxiety. Mice from both *Igf2* knockout models exhibited heightened discriminative accuracy in the 5-choice serial reaction time task, but *Igf2*-P0 KO mice also showed enhanced impulse control. Real-time qPCR revealed differential expression of both GABA- and 5-HT-ergic receptor subtypes in the hippocampus of *Igf2*-P0 KO mice, which may

correlate with the observed stress and anxiety related phenotypes. Moreover, alternate splicing of the 5-HT_{2C} receptor in the striatum could underlie the increased impulse control shown by *Igf2*-P0 KO mice. While the findings of elevated anxiety levels among the *Igf2*-P0 KO mice are in line with previous work on humans and animals, the heightened attentional performance and impulse control is entirely novel and highlight the importance of further research on the relationship between early life adversities and later ADHD risk.

List of content

CHAPTER VIII:

List of tables

List of figures

Chapter I

General Introduction

This thesis investigates the effects that the intrauterine environment may have on function in later adult life. This relationship, termed 'foetal programming', has been demonstrated to be a significant factor in a number of physiological conditions, most notably the metabolic syndrome, exemplified by adult onset (type-2) diabetes and obesity. Moreover, there is accumulating, but limited, evidence from human literature that this concept may also be of relevance to psychiatric disorders and behavioural dysfunction (e.g. attention deficit hyperactivity disorder (ADHD) and schizophrenia); this evidence is based on a range of different indices of early growth impairments such as low birth weight (LBW, 2500g >) or born small for gestational age (SGA). There have been numerous attempts to model these types of effects in animal models using different manipulations of the foetus or maternal nutrition, with various degrees of success and replicability. Despite varying approaches, many of these studies have highlighted a key role for hormonal systems, most notably the hypothalamic-pituitary-adrenal (HPA) and insulin-like growth factor (IGF) axes, in mediating these programming pathways.

1.1 Foetal programming

The tissues and organs of mammals and other species undergo a number of sensitive stages of development, known as critical periods, which are highlighted by phases of rapid cellular growth, proliferation and differentiation (Rice and Baron Jr., 2000). The critical periods vary between different organisms and programming can occur at different stages in life, including conception, foetal life, infancy and adolescence (Harding, 2004), and in terms of the development of the central nervous system (CNS), critical periods of growth during intrauterine life coincide with the stages of neuro- and glio-genesis, neuronal and glial migration, as well as synapse formation (Rice and Baron Jr., 2000). During these critical periods, stimuli or insults from the extra-foetal environment can have enduring effects on the structure, function or developmental schedule of the organism, a process which has been termed,

programming (Lucas, 1991). For instance, there is compelling evidence from epidemiological and animal work, demonstrating that adverse environmental factors acting early in foetal life, play a major role in determining a wide range of chronic diseases and disorders in adulthood (Barker, 1997). Moreover, whereas much of the earlier epidemiological observations focused on the metabolic and cardiovascular sequelae of this foetal-environmental interplay (McMillen and Robinson, 2005; Barker, 2004), more recent work indicates that the long-term consequences of adverse intrauterine environment may also involve brain and behavioural functions (Schlotz and Phillips, 2009). Although a host of environmental factors have been identified with a role in determining foetal growth rate (e.g. maternal stress and substance abuse, hormonal milieu and infection during pregnancy), a nutritional explanation for the relationship between intrauterine growth restriction (IUGR) and later disease, has been strongly advocated on the basis of current evidence (Harding, 2004; Hales and Barker, 2001; Lucas, 1991).

Over the last 20 years, the contribution of foetal programming to disease in later life has been a subject of heightened attention, and has attracted the interest of geneticists, physiologists, evolutionary biologists and psychologists. Furthermore, the work in this field of research has caused a major shift in the understanding of the aetiology of many common diseases. More specifically, the evidence from this work suggests that while most disorder are determined by genetic predisposition and/or postnatal environment and lifestyle, events occurring early in foetal life may also play a paramount role in the susceptibility for some these disorders. Unsurprisingly, gene-environment interplay is fundamental to the concept of programming, and in recent years the growing field of nutritional epigenomics has provided an important insight to the possible biological mechanisms that may underlie these interactions.

1.1.1 Evidence for foetal programming from epidemiological studies

The notion that intrauterine life events and maternal well being during gestation may have an enduring effect for the development of the child was generated on the basis of seminal work by Rose (1964). This study indicated a link between early life environment and later health outcome whereby familial patterns of coronary heart disease correlated with the survival rate of siblings and parental mortality. Further studies showed that infant mortality was geographically correlated with

arteriosclerotic heart disease in people aged between 40-69 years (Forsdahl, 1977) and similar geographical relations between infant mortality and adult coronary heart disease were subsequently reported by Barker and Osmond (1986). More recently, extensive data from human epidemiological studies across diverse populations have indicated that aberrant foetal development influences susceptibility to metabolic and cardiovascular diseases. For example, in a longitudinal study, with over 36,000 men and women from Hertfordshire, UK, results showed that people that had either LBW or reduced weight beyond one year of age, were at an increased risk of developing coronary heart disease (Barker, Osmond, Winter, Margetts and Simmonds, 1989). This pattern has been established for other components of the metabolic syndrome, such as obesity, stroke, hypertension, glucose intolerance and hyperlipidemia, in a number of subsequent large epidemiological studies (Leon et al., 1996; Martyn, Barker and Osmond, 1996; Hales, Desai, Ozanne and Crowther., 1996; Barker, Martyn, Osmond, Hales and Fall, 1993; Barker, et al., 1993). Consequently, Hales and Barker (Hales et al., 1991) formulated the foetal origins of adult disease hypothesis (FOAD, also known as 'the developmental origins of adulthood disease' (DOAD), the 'thrifty phenotype hypothesis' or the 'Barker hypothesis'); briefly, this idea attributes to poor nutritional environment a causal role in the relationship between compromised foetal growth and subsequent metabolic and cardiovascular disease susceptibility (Hales and Barker, 2001 and 1992). That such a relationship exists between later health and early life adversity may not be surprising when considering the rate of cell growth and differentiation that takes place *in utero* and during the early neonatal period, amplifying the impact of any adverse effects during these phases of life.

1.1.2 Genes versus nutrition

An earlier theoretical paradigm known as the 'thrifty genotype hypothesis' was postulated by Neel (1962; cited in Prentice, 2005) based upon the aetiology of large babies born to diabetic mothers and related to insulin resistance. According to this hypothesis, natural selection operating during the early stages of human evolution, could have produced a selection of genes that confer an efficient metabolic state and energy conservation for periods of time when food sources are scarce and food intake irregular. Furthermore, while such metabolic adaptation would enhance survival of the organism in times of food shortage, these alterations could be

detrimental during periods of over-nutrition and possibly lead to diseases in adulthood; a process thought to underlie the current trend towards increased incidence of type-2 diabetes (Prentice, 2005). Consistent with this idea, mutation of the glucokinase gene leads to both IUGR and postnatal hyperglycaemia (Hattersley et al., 1998) and the class III allele of the variable number of tandem repeat locus of the insulin (*INS*) gene has also been associated with increased foetal growth, as well as with insulin resistance, diabetic hypertriglyceridemia, atherosclerosis, cardiovascular diseases and type 2 diabetes (Mitchell et al., 2004).

However, while the aforementioned studies suggest that genetic predisposition could explain the relationship between IUGR and later physiology in some cases, there is substantial evidence in support for the environmental programming of later health from twin studies or where pregnant mothers experienced severe famine. In a Danish twin-study investigating type 2 diabetes among discordant monozygotic twins, results showed that the diabetic twins had a significantly lower birth weight relative to normoglycemic co-twins (Poulsen, Vaag, Kyvik, Jensen and Beck-Nielsen, 1997). Also, in an earlier study the occurrence of type II diabetes among first degree relatives was found to be greater within, than between, generations, a fact more suggestive of environmental than genetic influences for a nutritional origin of later disease (Beaty, Neel and Fajans, 1982).

An important role for environmental influences is further validated when the evidence from a number of famine studies is considered. In a landmark study carried out by Ravelli, Stein and Susser (1976) the long-term effects of intrauterine exposure to maternal malnutrition in a large sample of people born in Amsterdam at the time of the Dutch winter famine during the Second World War was investigated. The findings revealed a significantly increased prevalence for obesity among those exposed to under-nutrition in early gestation, than those malnourished later in pregnancy or those who were not malnourished at all. Moreover, subsequent findings from follow-up studies using the Dutch winter famine victims have reported significantly greater prevalence of impaired glucose tolerance (Ravelli, van der Meulen and Michaels, 1998) and coronary heart disease (Ravelli, van der Meuelen, Osmong, Barker and Bleker, 1999) following famine in early gestation, whereas famine exposure during mid-gestation has been associated with elevated risk of obstructive airways disease (Lopuhaä et al., 2000). The Dutch winter famine victims suffered from low maternal protein, carbohydrate and fat intake during the critical phase in development,

suggesting that the nutritional balance of the maternal diet may have significant and selective effects on foetal development and programming. In fact, there is some evidence that the maternal intake of fruit and vegetables during pregnancy, is positively correlated with birth weight and glucose tolerance in the offspring (Rao et al., 2001; Yajnik, 2004), further demonstrating the importance of nutrients (such as folic acid, glucose, triglycerides, cholesterol) in normal development and subsequent health.

In the context of the above studies conducted in humans, as well as findings from animal models (see below), there is substantial evidence indicating that environmental agents can independently impact on gene expression and lead to changes in physiological and morphological phenotypes, related to metabolic and cardiovascular disorders. Nevertheless, it is probable that at least in some cases, genetic predispositions act in concert with environmental agents to bring about changes in later disease vulnerability. For instance, a polymorphism in the peroxisome-proliferator-activated receptor γ2 receptor gene, which is involved in the development and metabolic function of adipose tissue, has been found to modulate insulin resistance susceptibility of people born SGA (Eriksson et al., 2002). Similar interactions with foetal growth have been described for insulin resistance with polymorphisms in angiotensin-converting enzyme, plasma cell glycoprotein 1, as well as in vitamin D receptor genes (see Godfrey, Cameron and Hanson, 2006 for review).

1.1.3 Evidence from animal models

Human epidemiological data has gained experimental support from findings in a variety of animal models, in which the impact of nutritional constraints on long-term metabolic and cardiovascular function has been investigated (Reusens, Kalbe and Remacle, 2004; Langley-Evans, 2004a). Surgical ligation of one of the uterine horns of a pregnant guinea pig to reduce foetal nutrition induced IUGR and subsequent significantly increased blood pressure when tested in young adulthood, relative to littermates derived from the other (intact) uterine horn (Persson and Janson, 1992). Elevated systolic blood pressure has also been reported in the offspring of rat dams that were fed protein restricted diet during gestation (Langley-Evans, Welham and Jackson, 1999; Langley-Evans, Gardner and Jackson, 1996; Langley-Evans and Jackson, 1994; Langley-Evans, Phillips and Jackson, 1994), but this cardiovascular

vulnerability has been found to be greatest when the maternal exposure to the low-protein diet occurs during the last week of gestation (Langley-Evans, Welham, Sherman and Jackson, 1996).

Furthermore, IUGR by nutritional restriction during foetal life has also been demonstrated to have numerous effects on glucose metabolism and insulin action in later postnatal life. For example, offspring of mothers fed low-protein diet during gestation have been reported to have selective insulin resistance (Ozanne, Dorling, Want and Petry, 2000; Ozanne, Wang, Dorling and Petry, 1999), glucose intolerance (Hales, Desai, Ozanne and Crowther, 1996) and diabetes (Petry, Ozanne and Hales, 2001), and uterine artery ligation has been demonstrated to lead to a reduction in foetal pancreatic β-cells (De Prins and Van Assche, 1998). Moreover, perturbations in the insulin-like growth factor (IGF) axis have been reported in foetuses that have been subjected to maternal malnutrition, in terms of decreased heptocyte cellular proliferation and insulin-like growth factor-I (IGF-I) production (El Khabatti, Gregoire, Remacle and Reusens, 2003), and decreased pancreatic insulin-like growth factor-II (IGF-II) mRNA expression (Petrik, Arany, McDonald and Hill, 1998).

1.1.4 Summary

Taken together, there is clear evidence from both epidemiological data and animal models that intrauterine nutrition can influence adult susceptibility to metabolic and cardiovascular disorders, additionally; genetic background may also play an important role. Moreover, animal models of nutritional restriction suggest that the IGF axis might play a central role in modulating foetal programming of this heightened disease risk. In recent years there has been a significant shift of focus in the research of foetal programming research, with an increasing number of studies examining the effects of early life environment on mental health in later life (Schlotz and Phillips, 2009; Connors et al, 2008; Kajantie, 2006; Breslau, 1995).

1.2 Foetal origins of mental health

1.2.1 Evidence from epidemiological studies

In case-control studies, children of impaired foetal growth have repeatedly been described to have heightened risk of cognitive deficits (Shenkin, Starr and

Deary, 2004), anxiety-related disorders (Lundgren, Cnattingius, Jonsson and Tuvemo, 2001; Nilson, Nyberg and Östergren, 2001), learning disabilities (Johnson and Breslau, 2000) and ADHD (Breslau et al., 1996), relative to children of normal birth weight. Moreover, impaired performance in tasks of executive function and perceptual motor skills (Taylor, Minich, Klein and Hack, 2004; Vicari, Caravale, Carlesimo, Casadei and Allemand, 2004; Strauss, 2000) have been reported in adolescents of LBW (generally taken to be indicative of gestational compromise), but these adults have also been shown to have a greater vulnerability for early drug use (Chilcoat and Breslau, 2002).

Following up the previously reported metabolic and cardiovascular sequelae of maternal malnutrition during the Dutch winter famine of World War II, Susser and colleagues (1996) showed a two-fold increase in risk of schizophrenia in adults that were most severely malnourished during gestation. A similar pattern for risk of schizophrenia was reported in a Chinese cohort that underwent a severe famine several years later (St. Clair et al., 2005). In other follow-up studies of the Dutch famine cohort, exposure to famine during the first and second trimester was associated with an elevated risk for antisocial personality disorder (Neugebauer, Hoek and Susser, 1999), whereas those exposed to famine during the second or third trimester were at greater risk of being diagnosed with mood disorders in later life (Brown, van Os, Driessens, Hoek and Susser, 2000). Taken together, these epidemiological findings provide basic support for foetal programming of later psychiatric disease susceptibility, by changes in early nutrition.

1.2.2 Evidence from animal models

Although human epidemiological data supporting a link between early nutritional origin and adult psychiatric vulnerability has only begun to emerge in the last 15-20 years, the experimental evidence for a relationship between brain function and early nutritional provisioning dates back much earlier, and in fact precedes the research on metabolic and cardiovascular effects. During the mid 1960's a number of research groups began to perform experiments in animal models to explore the effects of early life malnutrition on subsequent cognitive function and behaviour (Scrimshaw, 1967). Some of the initial findings showed that rats, piglets and puppies that were born to well nourished mothers but subsequently placed on protein deficient diet

developed motor impairments after only four days (Platt, Heard and Stewart, 1964 and Platt, 1962, cited in Scrimshaw, 1967). Furthermore, adult rats that were weaned prematurely (i.e. postnatal day 15) showed a greater delay in learning, and were more behaviourally inhibited in a simple learning paradigm (i.e. made fewer responses), relative to rats that underwent weaning at the usual age of 30 days (Nováková, Faltin, Flandera, Hahn and Koldovskỳ, 1962). Since then, an extensive amount of data has accumulated on the behavioural consequences of suboptimal nutrition availability during gestation, lactation and/or early postnatal life using a number of different animal models.

This research has consistently described cognitive impairments, as well as heightened anxiety and motivation in animals subjected to nutritional deprivation during lactation or pre-weaning periods (see Strupp and Levitsky, 1995 for review). In contrast, studies investigating the effects of nutritional insults occurring during intrauterine life, on adult behaviour, have yielded contradictory results. For example, offspring of protein restricted rat dams showed poorer reversal learning performance on the water-escape version of a T-maze apparatus (Villescas, Van Marthens and Hammer, 1981). However, in an operant version of the task where rats had to learn a new stimulus-reward pairing whilst ignore a previously learned stimulus-reward pairing, no differences were observed at baseline configurations, and in fact, superior performance was observed in the prenatally malnourished group of rats when tested at a heightened level of difficulty (Tonkiss and Galler, 1990). Similarly, offspring of protein restricted rat dams have shown impaired response acquisition on a task of differential reinforcement of low rate schedule (Tonkiss, Galler, Formica, Shukitt-Hale and Timm, 1990a). Rats that were exposed to food deprivation during gestation adapted less well to shifts in reinforcement schedules, when tested on a concurrent variable-interval paradigm (Landon et al., 2007). However, in contrast, there was no effect of nutritional deprivation during foetal life on memory and learning when tested in the Morris water-maze and fear conditioning paradigm (Gilbert, MacPhail, Baldwin, Moser and Chernoff, 2010). A similar absence of impairment in the Morris water-maze task was noted earlier in rats that suffered intrauterine protein malnutrition (Tonkiss, Schultz and Galler, 1994). Thus, the effects of gestational malnutrition in rats on later cognitive performance are not clear cut but nonetheless a number of significant effects have been reported.

A similarly confused picture emerges when the effects of foetal malnutrition on later stress and anxiety states are considered. Several studies have described heightened anxiety levels among adult rats that were exposed to either calorie or protein restriction during gestation when tested in ethologically based conflict tests of anxiety (Levay et al., 2008; Jaiswal, Upadhyay, Satyan and Bhattacharya, 1996) or in a test of stress responsiveness (Erhard, Boissy, Rae and Rind, 2004). Conversely, other studies have either not found any effect of intrauterine under-nutrition on stress- or anxiety-related behaviour (Simitzis et al., 2008; Whatson and Smart, 1978), or have even demonstrated anxiolytic effects (i.e. less anxiety) on adult behaviour following nutritional restriction during intrauterine life (Almeida, Tonkiss and Galler, 1996a; Almeida, Tonkiss and Galler, 1996b). Perhaps the only relatively consistent behavioural effects of intrauterine nutritional restriction have been noted in relation to parameters of motivation. For example, increased lever-pressing rates in order to receive rewards was observed in adult rats that were born from underfed dams (Smart, Dobbin, Adlard, Lynch and Sands, 1973) or from dams fed a low-protein diet during pregnancy (Tonkiss et al., 1990b). Similar results are obtained in a number of other studies employing both appetitive and aversively motivated experimental paradigms (see Strupp and Levitsky, 1995 for review). Overall, while there is considerable evidence from animal models suggesting that intrauterine malnutrition might lead to heightened motivation in later life, the direction and magnitude of effects of such early nutritional insults on later cognitive function and emotional state remains far from clear.

1.2.3 Summary

In light of findings from the human epidemiological studies, it is clear that the long-term effects of impaired foetal growth are not confined to metabolic and cardiovascular sequelae, but may also include behavioural and psychiatric functions. Previous work from animal models suggests that early life nutrition might play a role in establishing the relationship between IUGR and later mental health. Nevertheless, the great discrepant findings from the animal work, suggests that the aetiology of developmental programming of the CNS might be particularly heterogeneous and prone to postnatal environmental influences.

1.3 Foetal programming of brain development

It has now become widely accepted that interruption of development during intrauterine life can result in abnormalities of the brain (see Rice and Barone Jr., 2000). Furthermore, nutritional inadequacy has been advocated as the principal nongenetic component that influences brain development (Morgane, Mokler and Galler, 2002), as nutrients are essential for basic developmental programmes such as; neuronal growth and proliferation, neuronal and glial migration, myelinisation, and synaptogenesis (Georgieff, 2007; Morgane et al., 2002). Nutritional deficiency can also compromise the catalytic activities of enzymes, protein synthesis and structure that are critical for neuronal formation and tissue organisation (Langley-Evans, 2004b; Morgane et al., 2002). Whereas previous research on early life malnutrition has described reductions in later head circumference, brain weight and cerebral cell number in humans (Winick, 1969), the current understanding of the intrauterine effects of nutritional inadequacy on cerebral development and function, stems mainly from research in animal models.

1.3.1 Foetal programming of general brain morphology

Work with animal models using the imposition of low-protein diets during gestation in rats have demonstrated irreversible reductions in total brain weight, DNA and protein content, and in various cerebral lipid concentrations (Chase, Dabiere, Welch and O'Brien, 1971; Zamenhof, Van Marthens and Margolis, 1968). Other studies examining cerebral morphology in more detail have reported reductions in neuronal myelation (Bourre, Morand, Chanez, Dumond, and Flexor, 1981), dendritic branching of occipital pyramidal neurons (Cordero et al., 1986) and cortical thickness (Clark, Zamenhof, Van Marthens, Granel and Kruger, 1973) in adult rats that were gestationally malnourished. In addition, enlarged ventricles and reduced cortical, striatal and hippocampal volumes were observed in adult guinea pigs which experienced placental deficiency, induced by artery ligation (Mallard, Rehn, Reese, Tolcos and Copolov, 1999). Although there is evidence that brain weight/size is protected relative to the overall body weight and other organs (Ahmad and Rahman, 1974; Tizard, 1974; Dobbin and Widdowson, 1965), some parts of the brain may be more susceptible to environmental insults during early life, such as the hippocampus (Morgane et al., 2002).

1.3.2 Foetal programming of hippocampal circuitry

Compelling evidence from animal models shows that the volume of the hippocampus is markedly, and specifically, reduced as a result of early life environmental insult and/or nutritional deficiency (Mallard et al., 1999). This has highlighted the neural circuitry in this area as an important target in these manipulations. More detailed examination of the neuronal circuitry of this brain region has shown reduced numbers of pyramidal neurons in the CA1 subfield of the hippocampus in guinea pigs, which were IUGR as a result of uterine horn ligation of their dam (Mallard, Lolieger, Copolov and Reese, 2000). In rats exposed to intrauterine protein restriction, a significant decrease in somal size, basal dendritic branching and spine density in CA3 pyramidal neurons of the hippocampus (at day 220) (Díaz-Cintra, Carcí-Ruiz, Corkidi and Cintra, 1994) and asymmetrical synapse reduction between mossy fibre granule neurons and the CA3 pyramidal neurons (Granados-Rojas et al., 2002) have been reported. Increases in the number of granule cells in the dentate gyrus in protein malnourished rats (King et al., 2004; Nunes, Liptákova, Velísková, Sperber and Moshé, 2000) highlights the fact that different sub-regions of the hippocampus may be differentially affected by such manipulations.

In line with the prevailing evidence of morphological differences in the hippocampus of rats that underwent intrauterine malnutrition, a number of studies have demonstrated alterations in synaptic plasticity. More specifically, changes in the maintenance and magnitude of long-term potentiation (LTP). Thus, intrauterine protein or micronutrient (zinc) deprivation has been shown to alter both the time course and the magnitude of LTP in the dentate gyrus of adult rats (Bronzino, Austin La France, Morgane and Galler, 1996) and elevate rates of miniature inhibitory synaptic currents in CA1 (Luebke, St John and Galler, 2000) and CA3 pyramidal neurons (Chang, Galler and Luebke, 2003). Reductions in hippocampal N-methyl-D-aspartate (NMDA) receptor expression, implicated in the maintenance of hippocampal LTP (see MacDonald, Jackson and Beazely, 2006 for review, Chowanadisai, Kelleher and Lönnerdal, 2005) have also been described in the rat brain following intrauterine zinc deficiency. Moreover, rats exposed during gestation to a diet supplemented by excess choline, the precursor of the neurotransmitter acetylcholine, showed an attenuated LTP induction threshold in the CA1 region of the hippocampus (Pyapali, Turner, Williams, Meck and Swartzwelder, 1998).

1.3.3 Foetal programming of hippocampal neurotransmitter systems

The morphological and electrophysiological changes in the hippocampus resulting from intrauterine nutritional insults are paralleled by alterations to a number of neurotransmitter systems. Electrical stimulation of cultured hippocampal slices, derived from the brains of intrauterine protein malnourished rats, resulted in the long-term increase in the efflux of dopamine and its metabolites (i.e. 3,4–dihydroxy-phenylacetic acid (DOPAC) and homovanillic acid), decreased noradrenaline and its precursor tyrosine, and decreased serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA, Chen, Turiak, Galler and Volicer, 1995). Moreover, *in vivo* microdialysis studies targeted at the hippocampus of intrauterine malnourished rats, showed a significant decrease in 5-HT release when either exposed to mild electrical stimulation or when administered benzodiazepine agents, even though these rats had elevated basal levels of 5-HT in comparison to well-nourished rats (Mokler, Galler and Morgane, 2003; Mokler, Bronzino, Galler and Morgane, 1999).

In light of these findings, Mokler and colleagues (2003) proposed that intrauterine malnutrition might invoke an altered stress response via differential 5-HT modulation of inhibitory GABA (γ-aminobutyric acid) interneurons. Indeed, such anatomical and/or functional alterations in the 5-HTergic inputs that regulate the inhibitory activity of GABA neurons, could conceivably account for the LTP impairment and the heightened inhibitory signalling, mentioned previously. Consistent with this view, chronic protein restriction during gestation in rats has been found to reduce both the number of hippocampal GABAergic interneurons (Andrade and Paula-Barbosa, 1996; Díaz-Cintra et al., 2007) and hippocampal $GABA_{\alpha 1}$ receptor subunit expression, while $GABA_{\alpha 3}$ receptor expression was found to be up-regulated (Steiger, Alexander, Galler, Farb and Russek, 2003), along with lower cerebral basal GABA efflux (Del Angel-Meza, Ramíres-Cortes, Adame-González, González, Burgos, Beas-Zárate, 2002).

1.3.4 Summary

In summary, the research reviewed in this subsection indicates that undernutrition during intrauterine life can have enduring consequences on brain development and later brain morphology and neurophysiology. While the effects of

nutritional programming encompass relatively broad alterations in brain anatomy and involve alterations of numerous neurotransmitter systems, there are data which highlight both the structural and functional vulnerability of the hippocampal region, particularly in terms of the modulatory reciprocal circuitry of the 5-HT- and GABAergic systems. Importantly, the findings from the animal models of intrauterine malnutrition, are moreover broadly in line with results from studies of children with IUGR, which have described reductions in both cortical gray matter (Spencer et al., 2008; Martinussen et al., 2005; Tolsa et al., 2004) and hippocampal volume (Isaacs et al., 2000).

1.4 Programming of the placenta via functional alterations of the IGF and HPA axes

The placenta is the first organ to develop during mammalian embryogenesis and is responsible for the supply of vital nutrients to the growing foetus (primarily glucose, lactate and amino acids), disposing of harmful waste materials, and regulating the exchange of gases between the mother and the foetus. The placenta has the key role of integrating signals from the foetus and the mother in effort to accommodate the maternal substrate supply to foetal demand and at the same time meeting its own metabolic requirements. Therefore, successful placental development is of paramount importance for the optimal growth, maturation, and thus the survival of the embryo or foetus and concomitant formation of the adult. However, placental development, and in turn placental efficiency, is largely determined by nutritional availability (Fowden, Ward, Wooding, Forhead and Constância, 2006). Hence, significantly reduced maternal concentrations (i.e. from the umbilical vein) of some of the most essential amino acids have been noted in cases of IUGR (Cetin et al., 1996). The reduction in placental efficiency involves alterations in both foetal and placental gene expression, particularly via epigenetic modifications; such as altered DNA methylation, which has been shown to have repressing effects on gene transcription (Dolinoy, Das, Weidman and Jirtle, 2007; Gallou-Kabani and Junien, 2005). Importantly, in recent years there has been accumulating evidence indicating that early nutritional perturbation might induce foetal growth retardation and later physiological sequelae, by causing severe and lasting alterations in some of the major

components of the endocrine system, especially in terms of hyperactivation of the HPA axis, in parallel with down-regulation in the activity of the IGF axis.

1.4.1 The IGF axis

The IGF signalling system has previously been shown to play a key role in foetal and placental development throughout gestation (Fowden, 2003). The IGF system consists of the IGF-I and IGF-II signalling peptides, which are released from the foetal pancreas following exposure to glucose and amino acid stimulation (Rees, Wiznitzer, Le, Homko, Behrman and Martin, 1994). The mitogenic IGF-I and –II peptides are structurally homologous to insulin, and promote cellular differentiation, migration, aggregation, and inhibit apoptosis (Han and Carter, 2000). There are two receptors, which have unrelated structural features and binding properties: IGF type I and type II receptors (IGF-IR and IGF-IIR, respectively). IGF-IR mediates the majority of the biological actions of both IGF-I and IGF-II, although the insulin receptor also displays agonistic binding affinity for both peptides, but to a lesser degree. IGF-IIR functions as an antagonist to IGF-II activity. In addition to the two receptors, there are six IGF binding proteins (IGFBPs-1-6) that have been identified to date. The IGFBPs are found in both foetal plasma and tissue, and can either enhance or suppress IGF activity. The IGFBPs that are of the membrane-bound form (IGFBP-3 and -5) usually up-regulate IGF activity by chaperoning the IGFs to receptor sites and prolonging the half life of activity, whereas the soluble forms of IGFBPs (IGFBP-1,-2 (context dependent), and -6) inhibit IGF action (O'Dell and Day, 1998).

1.4.1.1 The role of IGF-I and IGF-II in foetal and placental growth

The IGF-I and IGF-II polypeptides are encoded by the *IGF1* and the *IGF2* genes respectively, which are both expressed in foetal and placental tissue (Fowden, 2003). Expression of the *IGF1* gene is biallelic, whereas the *IGF2* gene is imprinted and therefore monoallelically expressed in a parent-of-origin dependent manner (maternally silenced/paternally expressed). In gene manipulation studies, disruption of either *Igf1* or *Igf2* gene expression in mice has been shown to result in a considerable deficiency in foetal growth, to approximately 60% of normal birth weight (Baker, Lin, Robertson and Efstratiadis, 1993; DeChiara, Robertson and Efstratiadis, 1990, respectively). However, a simultaneous deletion of both the *Igf* genes produced far

more severe growth retardation, to only about 30% of normal birth weight (Efstratiadis, 1998). A similar severity in growth retardation was observed following suppression of the IGF-I receptor gene (*Igf1r*), notably in line with its binding properties for both the IGF-I and IGF-II peptides (Baker et al., 1993). Conversely, IGF-II up-regulation induced either by biallelic expression the of *Igf2* gene (due to loss of imprinting) or by suppressing the expression of the IGF-II clearance receptor gene (*Igf2r*), has been found to result in foetal overgrowth (Lau et al., 1994; Ludwig et al., 1996). Placental growth does not seem to be affected by manipulation of *Igf1* or *Igf1r* genes in mice, whereas when the *Igf2* gene was either abolished from all foetal and placental transcripts (i.e. *Igf2*-Null mice) (DeChiara et al., 1990), or a from a placental exclusive transcript of the labyrinthine trophoblast (i.e. *Igf2*-P0 mice) (Constância et al., 2002), the placenta is growth retarded by 30-40%.

An important role for the IGF axis in foetal growth regulation in humans has been proposed on the basis of findings from studies in which circulating levels of IGF-I were correlated with several different parameters of growth including birth weight, birth length, placental weight, and ponderal index (Klauwer el al., 1997; Osorio et al., 1996). In addition, IUGR in children born at term has also been related with lowered maternal IGF-II serum levels (Holmes et al., 1997), as well as reduced *IGF2* expression in the placenta (McMinn et al., 2006). On the other hand, foetal body weight at term has been positively correlated to plasma IGFBP-3, whereas an inverse association has been observed between foetal growth and IGFBP-1 (Kajantie et al., 2001). Dysregulation of *IGF* expressions has been implicated in several developmental disorders. For instance, homozygous molecular defect in the *IGF1* gene has been noted to result in severe IUGR, along with sensory-neuronal and mental impairments (Camacho-Hübner, Woods, Clark and Savage, 2002). Furthermore, constitutive over expression of *IGF2* is among the causal factors for Beckwith-Wiedemann syndrome (BWS), an overgrowth disorder characterised by macrosomia (birth weight and length >90th percentile) and cancer predisposition; conversely, *IGF2* down-regulation is associated with Silver-Russell syndromic dwarfism (Demars et al., 2010).

1.4.1.2 Programming of the IGF axis

Several studies examining nutrient restriction during intrauterine life have demonstrated down-regulation of the IGF axis. For example, restriction in maternal food intake has been shown to lower foetal circulating concentrations of IGF-I and IGF-II and mRNA expression of IGF-I and IGF-II in both rats (Strauss and Takemoto, 1991; Stauss, Ooi, Orlowski and Rechler, 1991) and sheep (Bramfeld et al., 2000; Lee et al., 1997; Kind et al., 1995; Oliver, Harding, Breier and Gluckman, 1996), whereas elevated plasma levels of IGFBP-1 are a consequence of reduced nutrient availability induced either by maternal fasting (Osborn, Fowlkes, Han and Freemark, 1992), dietary restriction (Strauss et al., 1991) or unilateral artery ligation (Price, Roog, Stiles and D'Ercole, 1992). In addition to the links between the IGF axis and birth size mentioned previously (Holmes et al., 1997; McMinn et al., 2006; Kajantie et al., 2001), hypomethylation of the *IGF2* gene has been observed in the offspring most severely affected by the Dutch winter famine of the Second World War (Heijmans et al., 2008) which speculatively may reflect an epigenetic compensation in response to the nutritional deprivation experienced. Thus, there is evidence from both human and animal studies demonstrating the involvement of the IGF axis in intrauterine nutrition and foetal development.

1.4.2 Regulation of the IGF system by the HPA axis

There is strong evidence from human and animal work that the HPA axis is highly susceptible to nutritional insults during foetal life, and might be involved in modulating the effects of early nutrient restriction on IGF activity. The HPA axis represents a major part of the neuroendocrine system and plays a key role in mediating the stress response of the organism via excretion of glucocorticoid steroid hormones (Plotsky, Thrivikraman and Meany, 1993). In response to stressful stimuli, the hypothalamic neurons of the paraventricular nucleus synthesize and secrete corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), which in turn drive the synthesis and release of adrenocorticotrophin hormones (ACTH) from the anterior lobe of the pituitary gland. Subsequently, ACTH stimulates the adrenal cortex to produce and release corticosteroid hormones (corticosterone in rodents, cortisol in humans) which then, in sufficient concentrations, provide inhibitory feedback signals to the hypothalamus and pituitary gland via glucocorticoid and

mineralocorticoid receptors of the hippocampus, thus suppressing the production of CRH and ACTH (Kapoor, Petropoulos and Matthews, 2008). The glucocorticoid hormones are essential for the successful adaptation of an organism to acute physical stressors by ensuring adequate glucose availability for the brain and other vital tissues. Nevertheless, the release of glucocorticcoid steroids requires strict regulation, since excess glucocorticoid secretion can lead to various detrimental effects, particularly in terms of heightened risk for cardiovascular disease (Reynolds et al., 2010; Walker, 2007), the metabolic syndrome or its components (Kajantie, 2006), as well as notably impaired hippocampal development and function (Gallagher, Reid and Ferrier, 2009; Brown, Rush and McEwen, 1999).

1.4.2.1 Programming of the HPA axis

Epidemiological research assessing the relationship between the intrauterine environment and aberrant HPA function, have proposed alterations in corticosteroid secretion as one of the key deleterious consequences of poor foetal growth, although some discrepancy has been noted in previous findings. For instance, while numerous studies reported heightened basal cortisol levels among people of LBW (Lewitt, et. al., 2000; Philips et al., 2000; Philips et al., 1998) or born SGA (Cianfarani, Geremia, Scott and Germani, 2002; Houang, Morineau, Le Bouc, Fiet and Gourmelen, 1999), other studies have indicated lowered resting cortisol levels (Kajantie et al., 2007; Hng, Cheung and McLean, 2005) or following administration of the cortisol analogue dexamethasone (Kajantie et al., 2003; Kajantie et al., 2002). Moreover, these latter results are also bolstered by the fact that hypocortisolism has been demonstrated to underlie several stress-related conditions (see Heim, Ehlert and Hellhammer, 2000 for review). However, these discrepancies may be accounted for, at least in part, in terms of methodology and problems associated with accurate measuring of cortisol or grouping subjects by birth size alone. For example in a study carried out by Kajantie and colleagues (2006) elevated fasting cortisol concentrations in adulthood were observed in people born small but early (prior to 40 weeks of gestation), whereas the reverse was true for individuals born but small after 40 weeks gestation. In addition, three week food abstinence has previously been shown to result in a significantly elevated cortisol secretion in response to dexamethasone administration in human volunteers (Fichter and Pirke, 1986).

Extensive work from animal models employing intrauterine interventions of disrupted nutrition (as discussed previously) has demonstrated profound programming effects on the HPA axis. Maternal under-nutrition (only half of the daily food intake of control rat dams) during the last week of gestation increased both plasma corticosterone between embryonic days 19-21 and relative adrenal weight at term, and newborn rats displayed a reduction in ACTH circulation and lowered glucocorticiod and mineralocorticoid receptor expression in the hippocampus (Lesage, Blondeau, Grino, Breant and Dupouy, 2001). Similarly, rats subjected to a low-protein diet during intrauterine life showed blunted diurnal patterns of plasma ACTH concentrations, but also a persistent increase in glucocorticoid receptor capacity (Langley-Evans, Gardner and Jackson, 1996), and pigs of low birth weight have been shown to have a greater stimulated cortisol response and adrenal gland size, at three months of age (Poore and Fowden, 2003). Transient reductions in maternal caloric intake in sheep also results in a significantly greater ACTH response following administration of either CRH, AVP (Bloomfield et al., 2003), or insulin (Bloomfield et al., 2003; Edwards et al., 2001) in lambs, but these effects were not noted following a more prolonged exposure to calorific restriction to the ewe. However again, the findings from these and several other similar studies highlight that the precise implications of poor nutrition during intrauterine life on the function of the HPA axis is largely determined by the degree and duration of the under-nutrition (see McMillen and Robinson, 2005 for review).

1.4.2.2 Glucocorticoid regulation of IGF axis

During intrauterine development, there is a co-ordinated regulation of glucocorticoid synthesis and *IGF* gene expression. This has been demonstrated in a number of different ways including; the down-regulation of IGF-II transcription in the liver of neonatal rats (Levinovitz and Norstedt, 1989; Beck, et al., 1988) and the increased activity of IGF-inhibiting IGFBP-1 mRNA expression (Luo, Reid and Murphy, 1990) following administration of dexamethasone, and a reduction in IGFBP-3 expression in hepatic cells exposed to dexamethasone *in vitro* (Villafuerte, Koop, Pao and Phillips, 1995). Administration of naturally occurring corticosterioids and thyroid hormones have also been revealed to cause hepatic reduction in *IGF2* expression (Kitraki, Philippidis and Stylianopoulou, 1992) and similar reductions in

IGF2 expression have been found in ovine foetal adrenal glands when maternally exposed to either glucocorticoids or ACTH (Lu et al., 1994). These findings are in line with a previous proposal that the transient release of cortisol that usually occurs soon after birth might be responsible for the normal decline in IGF II transcription in early postnatal life (Dell, Ward, Shokrai, Madej and Engström, 1999; Dalle, Pradier and Delost, 1985). Therefore, it is possible that early life adversities (such as poor nutrition) could produce extensive alterations in the scale or onset of this developmental reprogramming of the endocrine system, with detrimental consequences for the organism in later life.

1.4.3 Summary

The endocrine activities of the IGF and the HPA axis have a central role in regulating foetal growth and development. These hormonal systems act as maturational and nutritional signals in intrauterine life, and control tissue accretion and differentiation in response to the prevailing environmental conditions surrounding the foetus (Fowden and Forehead, 2004). In particular, altered glucocorticoids secretion as a result of compromised placental function, is potentially pivotal in foetal programming, by altering the bioavailability of key hormones, and the cellular expression of receptors, enzymes, ion channels, transporters, and various cytoskeletal proteins in the foetal tissue (Fowden and Forehead, 2004). Moreover, glucocorticoids can act directly on gene expression and indirectly via other hormonal systems, such as the IGF system. The interplay between the IGF and HPA axis is likely to be at the nexus in the relationship between the intrauterine environment and later health.

1.5 Towards a new approach investigating the long-term effects of foetal programming and IUGR by nutritional deficiency

In view of the current evidence, it is evident that a range of environmental agents can exert adverse influences upon the early developmental trajectory of the CNS, by inducing epigenetic alterations in gene expression that ultimately lead to stable and sustained changes in cognitive and behavioural endophenotypes. However as noted previously, whereas the developmental programming of metabolic and cardiovascular complications has been well established across different species and

varying methods of nutritional interventions, the relationship between early foetal environment and later psychiatric health is less clear. Indeed, whilst a large number of psychiatric complications have been connected with environmental adversities during foetal life, previous epidemiological and animal model work has remained unsuccessful in dissociating the varying effects of different environmental factors on later cognition and behaviour, or in addressing the marked discrepancies observed between findings. Some of the inconsistencies in these studies could stem from the heterogeneity inherent in neuronal development, composition and compensation, both across and within species. As such, it is currently difficult to address which of the later (adult) psychiatric complications, result specifically from poor intrauterine nutrition rather than other environmental agents.

1.5.1 Challenges of epidemiological studies

Even though much of the evidence associating foetal-environmental interplay with later psychiatric health risk is derived from epidemiological observation, most of these studies suffer from serious methodological challenges. The vast majority of human studies have relied exclusively on measurements of birth weight and relative size at gestation as indicators or surrogate markers of foetal growth and/or the intrauterine environment. However, the presumption that LBW or being born SGA reflects adverse intrauterine environment or foetal nutritional status, is highly questionable. For instance, this approach provides only a transient glimpse of the developmental trajectory occurring during foetal life, and neglects the potential different growth patterns by which the foetus might have arrived at weight of birth (McMillen and Robinson, 2005). Moreover, the overall foetal weight and size at birth is an amalgam of a number of different environmental influences, and whilst some of these might have causal relations with later psychiatric disease risk, other might not (Simmons, 2005). In fact, it is likely that many environmental agents acting during intrauterine life might exacerbate later disease risk without affecting birth weight. More fundamentally, the usage of birth weight and gestational size as a proxy for foetal growth, also fails to take into account individual differences; i.e. the fact that some of the infants may have actually reached their genetic growth potential and are merely in the lower end of the normal growth variation, whereas others might have

undergone foetal undernutrition and growth restriction, but still reached a birth weight within the normal range (McMillen and Robinson, 2005).

Another problematic issue raised concerning the interpretation of the effect of malnutrition on foetal growth restriction is the fact that maternal calorific and macronutrient intake often has relatively little impact on the birth weight of offspring, except at the extremes of food intake (Gillman, 2002). This could suggest that maternal nutrition does not necessarily correlate with foetal nutrition, such that the allocation of nutritional resources might be temporarily favoured towards foetal requirements at the expense of the mother. In spite of the attempts by studies using large famine cohorts to isolate and identify the particular sequelae of poor intrauterine nutrition on later health, it has been acknowledged that there are serious limitations to this approach. While these individuals were likely to have been exposed to under-nutrition during foetal life, it is difficult to rule out that any increased susceptibility to later psychiatric risk is in fact specifically due to the under-nutrition rather than other environmental factors. A famine exposed population was also likely to have been under serious stress, in relation to the famine, war and other accompanying hardships, and hence, it is possible that any observed detrimental effects on later adult psychiatric health was due to maternal stress rather than solely poor foetal nutrition (indeed, there is an abundance of evidence linking maternal stress with psychiatric disorder in later life, see Rice et al., 2010 for review). Moreover, it is also difficult to dissociate the effects of poor nutrition and the ingestion of other toxic substances, potentially used as alternative replacements of food sources (Brown and Susser, 2008).

1.5.2 Challenges of animal models

To substantiate the human epidemiological studies, extensive research efforts have concentrated on establishing animal models of foetal malnutrition and IUGR, as discussed in the previous sections of this chapter. Animal models provide better control over potential genetic and environmental confounds, improved analysis of the outcomes of these manipulations (in terms of pathology, neurology, and neurobiology), and the shorter life span of animals allows for longitudinal studies to be accomplished in a much shorter time frame. Thus, placental deficiency has been emulated using a number of different methodologies: uterine artery ligation (Sanders

et al., 2004; Houdjick, Engelbregt, Poop-Snijders and Delemarre-Vd Waal, 2000), uterine and umbilical artery embolism (Louey, Cock and Harding, 2003; Bloomfield, van Zijl, Bauer, and Harding, 2002; Block, Schlafer, Wentworth, Kreitzer and Nathaniels, 1990), carunclectomy (Butler, Schwartz and McMillen, 2002; Rees et al., 1998) and hypobaric hypoxia (Llanos, 2002; Gardner, Fletcher, Fowden and Giussani, 2001). These methods of inducing IUGR are very crude in nature, and could have severe and nonspecific morphological and physiological consequences, for both the foetus and the mother, and may represent the most severe examples of IUGR in the human population (e.g. hypoxia at birth).

A large body of work has also been attempted utilizing more moderate means of affecting foetal nutritional status among rodents and sheep, involving underfeeding dams (e.g. access to only 50% of normal food intake), and dietary manipulation by reduction of global calorific protein or iron content (Armitage, Khan, Taylor, Nathanielsz and Poston, 2004). However, as mentioned previously, much of this research has produced inconsistent findings, which may result from factors such as the variations in feeding regimens, dietary challenges and differences in the timing and duration of the maternal dietary interventions. Perhaps of greater importance is the fact that several studies have demonstrated that depriving dams of regular food intake or subjecting them to dietary imbalance has an effect on subsequent care-taking behaviour up to weaning which could lead to a range of problems for the neonates when adult. Thus, malnourished mother rats displayed greater amounts of exploratory and passive behaviour in the presence of their offspring during the suckling period (Crnic, 1976: Massaro, Levitsky and Barnes, 1974; Smart and Preece, 1973) and also demonstrated greater latency in pup retrieval (Crnic, 1976). Moreover, malnourished mother rats showed greater care-taking behaviour towards their foetally malnourished pups, than did normally fed mother rats (Massaro, Levitsky and Barnes, 2004; Galler and Tonkiss, 1991). Mothering style in rats, through the pre-weaning phase has also been shown to have profound effects on stress reactivity, measured in terms of differential behaviour and altered HPA function and also inherited mothering styles (Champagne, 2008; Champagne et al., 2008; Caldji, Diorio and Meaney, 2000). Moreover, these variations in maternal caretaking behaviour have also been shown to influence the epigenetic status of hippocampal glucocorticoid receptors, providing further evidence for the major effects that alterations in the postnatal environment may have on adult behaviour (Weaver et al., 2004).

1.5.3 Summary

In light of these findings, it difficult to conclude whether changes in cognitive and behavioural performance as an adult, are actually due to *in utero* nutritional deficiency rather than differential postnatal rearing environments (or other dam-pup or pup-pup interactions). Despite the range of experimental findings discussed in the previous sections relating to the use of animal models of poor intrauterine nutrition, many of the criticisms raised against these models have been largely ignored and hypotheses based on the results still persist unrefined. Furthermore, with the importance placed upon animal work in helping to understand human conditions (a key component of translational research) these limitations highlight the importance of the development of more subtle and less confounded animal models of nutritional and placental deficiency. One effective approach involves producing genetic models of placental deficiency that would enable the circumvention of other environmental confounds (i.e. maternal malnutrition, stress and altered care-taking) without employing gross physically invasive procedures. The development of such a genetic model is among the main purposes of the present thesis.

1.6 Aim of the thesis: utilisation of *Igf2*-knockout mouse models to investigate the effects of placental deficiency and IUGR on brain function and behaviour

The advances in targeted mutagenesis have provided an excellent opportunity to investigate the foetal programming effects of placental deficiency on later health outcome, for instance by manipulating placental and/or foetal expression of growth-regulating imprinted genes. The development of such models of placental deficiency is highly relevant to simulate conditions of foetal malnutrition in human pregnancy, especially in light of the recent accumulation of data indicative of dysregulated placental expression of imprinted genes in cases of IUGR and infants born SGA (Diplas et al., 2009; Apostolidou et al., 2007; McMinn et al., 2006). Furthermore, while several studies have adopted knockout (KO) and transgenic mouse models to assay the role of imprinted genes in foetal and placental development (Bressen et al., 2009), to our knowledge, none of these models have been used to investigate the long-term consequences of placental deficiency and/or foetal growth restriction for adult disease risk, in the area of brain function and behaviour.

Therefore, the main objective of the research described in the present thesis, is to employ mouse models where the imprinted gene *Igf2* has been deleted and investigate the effects of placental deficiency and/or foetal growth restriction, on subsequent brain function, cognitition and behaviour in adult animals. Two *Igf2*-KO mouse models; *Igf2*-P0 and *Igf2*-Null, will be used, which differ in terms of the tissue types in which *Igf2* expression has been ablated. This differential pattern of expression is achieved by specific deletion of one of the four promoter sequences of the *Igf2* gene. Thus, in the *Igf2*-P0 KO model initially described by Constância and colleagues (2002), the single placentally expressed transcript from the P0 promoter is suppressed, whereas transcripts expressed from the foetal P1-P3 promoters remain intact. This results in considerable deficiency in the growth of the placenta (only 76% of wild-type placenta weight) throughout mid-late gestation, along with complete lack of placental IGF-II and decreased placental permeability. In addition, *Igf2*-P0 KO foetuses show growth restriction and reach only 78% of wild-type weight at E19, followed by postnatal compensatory growth that is completed by 3 months of age. On the other hand, in the *Igf2*-Null KO mouse model where transcripts from all four of the *Igf2* promoters are disrupted, mice exhibit a more severe restriction in foetal growth, 40% of wild-type weight (DeChiara et al., 1990). Although, the placental weight is equivalently reduced as with the *Igf2*-P0 KO line, the *Igf2*-Null KO mice do not demonstrate any acceleration in early postnatal growth rate and remain small for life. Comparison of the different models will be of interest as the relative demands of the foetus and the ability of the placenta to function normally differ. Thus, the placenta of *Igf2*-P0 KO mice is of reduced size, yet the foetus is not affected by the gene deletion and therefore has 'normal' demands, whereas in the *Igf2*-Null KO model, although the placenta is smaller than wild-type, the foetus size is proportional and hence there is no mismatch in foetal demand and placental capacity (Angiolini et al., 2006).

In the present research, the performance of *Igf2*-KO mice when adult was compared to wild-type (WT) littermates on a wide variety of behavioural tests, as outlined in Chapter II. Since much of the previous work in both humans and animal models have highlighted the relationship between early life nutrition and anxiogenic behaviour in later life, it was of particular interest to examine the *Igf2*-KO mouse models on behavioural tests, sensitive to stress and anxiety-related phenotypes.

Furthermore, while there have been numerous studies reporting associations between low birth weight and ADHD symptoms, there has not been much experimental work carried out to investigate the effects of intrauterine malnutrition on postnatal attentional function and impulsivity, in animal models. Therefore, behavioural tests developed for examination of various behavioural aspects of working memory function, were also used to investigate the *Igf2*-KO mice on ADHD-related behaviour. Early postnatal growth and somatic indices will be described in Chapter III, along with initial behavioural characterization by behavioural batteries of general locomotor activity and stress reactivity. Further assessment of stress reactivity and anxiety-related phenotypes between the *Igf2*-KO mice and WT littermates are discussed in Chapter IV, whilst attention-related processes and facets of behavioural inhibition are the subjects of interest in Chapter V and Chapter VI. Finally, a preliminary molecular assay of some of the key neural substrates potentially underlying observed differences in behavioural phenotypes will be addressed in Chapter VII.

Based on the findings presented in this chapter of significant correlations between intrauterine malnutrition, behavioural and cognitive dysfunction and neurobiological changes, it is hypothesised that mice of the *Igf2*-KO models (both the *Igf2*-P0 and *Igf2*-Null lines) will show significant differences in behaviour, cognition and neurobiology to WT littermates. Moreover, these effects may be more prevalent in the *Igf2*-P0 KO model, where there is a discrepancy in the foetal demands and placental ability to provide nutrition, thus increasing the level of foetal stress. The findings from the current work will provide strong experimental support for the existence of psychiatric sequelae of foetal programming by placental deficiency and/or IUGR, and will shed important light on the discrepancy of past research in the field of foetal programming and suggest an alternative means of pursuing this important field of research.

Chapter II

General Materials and Methods

The purpose of this chapter is to provide descriptions of the procedures that were routinely performed throughout the course of work for 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 Professor Lawrence Wilkinson (PPL 80/1937). Work was carried out under the Home Office Personal License granted to Mikael Allan Mikaelsson (PIL: 30/7657).

2.1 Production of the *Igf2*-knockout mouse models

The *Igf2*-Null KO mouse model was produced using embryonic stem (ES) cell knockout (KO) technology, previously described by DeChiara, and colleagues (1990). In brief, a replacement vector containing two selectable markers; the bacterial neomycin-resistance gene (*neo*) and the herpes simplex virus (HSV) thymidine kinase gene (*tk*), was used in order to target the *Igf2* gene. These markers enabled the application of a positive-negative selection protocol, using drugs G418 and gancyclovir (GANC), which identifies DNA recipient cells whilst reducing the background of random integrants. This resulted in a significant enrichment for targeted clones (homologous recombinants). The targeting procedures involved isolating a phage λ clone carrying the mouse *Igf2* gene, but the fragments of the cloned *Igf2* gene and the transcriptionally competent *neo* and *tk* cassettes, were then used for the construction of the replacement vector. The *neo* cassette, replacing 0.25 kilobases (kb) of *Igf2* gene sequence in the region of the first coding exon (E2), was flanked by 1.6 and 8.3 kb of murine *Igf2* locus DNA, in terms of 5' and 3' respectively. On the other hand, the *tk* cassette was attached at the 3' end of the 8.3-kb fragment. Next, the linearized replacement vector DNA was introduced into recipient CCE ES cells by electroporation, the cell were seeded and selected on feeder layers of STO fibroblasts, and the CCE.33 cells were injected into host blastocysts obtained

from the mouse strain C57B1/6, and implanted in pseudo-pregnant 129 female mice to produce founder mice.

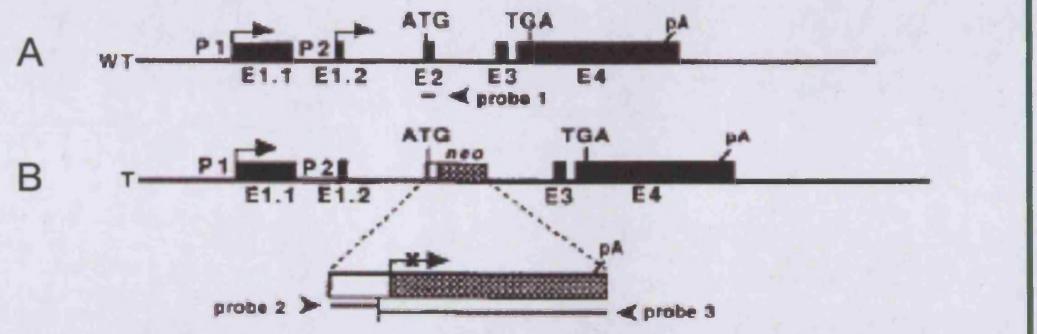


Figure 2.1: *Characterization of the Igf2-Null mutation, as indicated by restricion maps of the WT and targeted mouse Igf2-Null alleles.* ***(A)*** *The position of five exons (E1.1-E4; black boxes) of the WT Igf2 gene. P1 and P2 are promoters from which the alternate 5' noncoding exons E1.1 and E1.2 are initiated (arrows). The position of the initiation codon (ATG), termination codon (TGA), and major polyadenylation signal (pA) are shown.* ***(B)*** *The regon of E2 exon represented in the E2 riboprobe is indicated, along with enlargement of the cassette segment. In the targeted gene, in which only the Igf2 P1 promoter seems to be active, a neo cassette (1.15kb) has replaced exon E2 and about 0.1kb of the downstream intron (total of 0.25kb). The cassette segment, consists of the coding region of the neo gene (stippled box) and regulatory sequences (open box). The latter are a mutant polyoma enhancer and the herpes simplex viru tk promoter that are unable to initiate transcription (crossed-out arrow) of a ~0.9kb RNA when presented in the mutated gene. Also displayed is the position of the two neo riboprobes. Figure obtained from DeChiara et al., 1990.*

The *Igf2*-P0 KO model was derived by using 3.0-kb, 4.9-kb and 2.6-kb *Bam*HI genomic fragments derived from the *Igf2* locus of mouse strain 129/Sv origin as regions of homology, in order to generate a Cre-*loxP* targeting vector (Constâcia et al., 2000). Briefly, the *loxP site* of the targeting vector, was inserted 5' of exon U2, whereas a *loxP*-flanked HSV-*tk*-*neo*^{r} cassette was inserted 3' of the DMR1 region. The targeting vector was linearized at a unique *Xho*I site outside the area of homology and introduced into 1-2x10^7 CGR8.8 cells with a Gene Pulser at 500μF and 230V. Transfected cells were plated on gelatinized dishes pre-seeded with mitomycin-treated STO feeder cells. Following a 24 hour period, positive selection was performed in the presence of G418, and resistant clones were drawn and expanded into plates containing mitomycin-treated feeder cells. The neo^r cassette was removed by transiently transfecting Cre recombinase in DMR1-U2 *neo*^{r} cell lines with pMC-Cre. Finally, the DMR1-U2 neo^r cells were injected into host blastocyst of the mosue strain

C57B1/6, and implanted in pseudo-pregnant 129 female mice to generate founder mice.

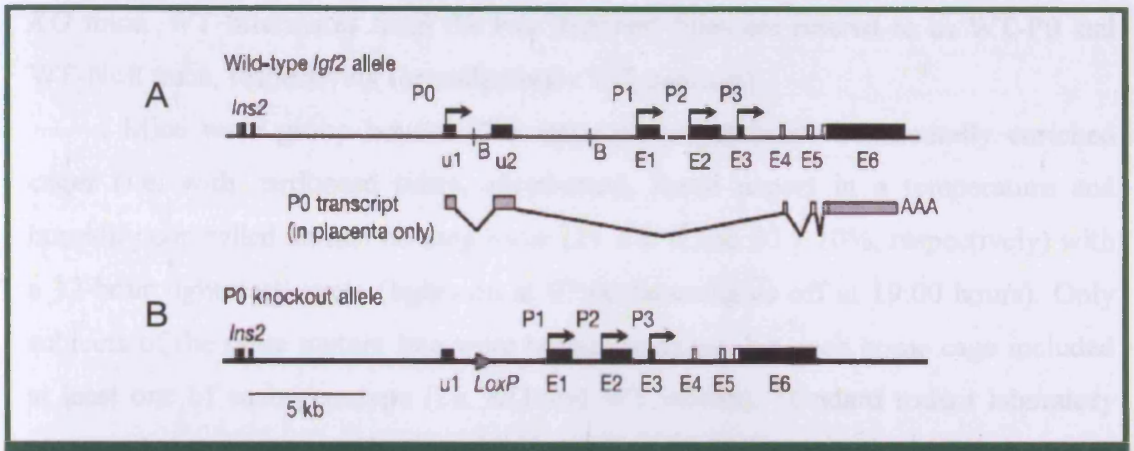


Figure 2.2: *Characterization of the Igf2-P0 mutation, as displayed by the genomic structure of the Igf2 gene in both WT's and KO alleles. The peptide coding region is in exon 4-6 (E4-E6, open boxes).* ***(A)*** *Promoters P1-P3 are transcribed in foetal and extraembryonic tissues. The P0 transcript is placenta-specific. All transcripts are spliced onto exon 4, leading to the same peptide.* ***(B)*** *A 5kb BamHI genomic fragment was replaced by a loxP site in the P0 KO allele. The deletion, which spans exon u2 and abolishes P0 transcription is 1.5kb and 2.1kb distant from P0 and P1 transcription sites, respectively. Figure obtained from Constância et al., 2002.*

2.2 Subjects and animal husbandry

The founder mice of the *Igf2*-P0 and *Igf2*-Null deletion lines were originally generated using the methodology described above (section 2.1), at the Laboratory of Development and Genomic Imprinting at the Babraham Institute, Cambridge, U.K.. Once the subjects were certified free from all common rodent pathogens, eight male subjects (at three to five months of age) were transported to the Behavioural Neuroscience laboratory of the School of Psychology, at Cardiff University. The mutant alleles in both lines were transmitted paternally by a heterozygous background strain of C57BL/6*129, and the breeding stock was established by crossing the C57BL/6*129 male subjects carrying the *Igf2* deletions, with CD1 female mice, resulting in offspring that were either heterozygous (*Igf2*-P0 or *Igf2*-Null KO) or wild-type (WT). Since the maternal allele of the *Igf2* locus is silenced due to imprinting regulation, it is the mutation transmitted along the paternal line that causes disruption in expression of the active paternal allele, thus the imprinted *Igf2* gene is expressed from neither paternal line (Constância et al., 2005). Throughout this thesis, KO mice

that lack *Igf2* expression from the *Igf2*-P0 only promoter are termed *Igf2*-P0 KO mice, whereas KO mice which had *Igf2* expression abolished from all four promoters are referred as *Igf2*-Null KO mice. In addition, these mice are collectively termed *Igf2-KO* mice. WT littermates from the two different lines are refered to as WT-P0 and WT-Null mice, respectively (or collectively WT controls).

Mice were 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 mutant line were housed together, but each home cage included at least one of each genotype (i.e. KO and WT mouse). Standard rodent laboratory chow and water were available *at libitum* unless otherwise stated. Home cages were cleaned and changed once a week, at approximately 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 mice were tested in the present research. Experimental animals were regularly monitored and weighed from birth for signs of ill health. Any mice showing signs of illness were immediately assessed by a Veterinarian and, if necessary, withdrawn from the experiment. Sentinel mice housed in the same environment as the experimental cohorts were, at intervals, assessed for evidence of variety of bacterial, viral and helminthic pathogens using the service at Surrey Diagnostics, U.K., and were always found to be uninfected (Table 2.1).

Table 2.1: *A list of the pathogens which the cohorts of experimental subjects were tested for.*

Viruses	Cases	Parasites	Cases
Minute virus of mice	0/1	Intestinal protozoa	0/1
Mouse hepatitis	0/1	Intestinal helminths	0/1
Pneumonia virus of mice	0/1	Athropods	0/1
Reovirus type III	0/1	**Bacteria**	
Mouse encephalomyelitis virus	0/1	Bordetella bronchiaseptica	0/1
Sendai virus	0/1	Campylobacter spp.	0/1
Epizootic diarrhoea of infant mice	0/1	Citrobacter rodentium	0/1
Mouse parvovirus	0/1	Clostridium piliforme	0/1
Mouse norovirus	0/1	Corynebacterium kutscheri	0/1
Lymphocytic choriomeningitis virus	0/1	Helicobacter spp.	0/1
Hantaan virus	0/1	Helobacter hepaticus	0/1
Ectromelia virus	0/1	Helicobacter bilis	0/1
Mouse cytomegalovirus	0/1	Helicobacter rodentium	0/1
Mouse adenovirus 1	0/1	Mycoplasma pulmonis	0/1
Mouse adenovirus 2	0/1	Pasteurellacae spp.	0/1
Polyoma virus	0/1	Salmonella spp.	0/1
K virus	0/1	Strectobacillus moniliformis	0/1
		Streptococcus pneumoniae	0/1
		B-haemolytic Streptococci	0/1
		Yersinia spp.	0/1

2.3 Standard PCR protocol

Mice were genotyped from DNA extracted from tail biopsies.

2.3.1 Tail lysis

A tail biopsy was collected from each mouse following weaning, at approximately four weeks of age. The collected tissue samples were placed in a 1.5ml micro-centrifuge tubes and were digested in 400μl of lysis buffer (0.2% SDS, 50mM Tris: HCl pH=8.0, 10mM EDTA, 100mM NaCl) along with 2μl of Proteinase K (Qiagen, Crawley, U.K.) with final concentration of 0.2mg/ml, at 55°C overnight.

2.3.2 DNA extraction

The 1.5ml micro-centrifuge tubes containing the digested tails were shaken briefly and spun in a centrifuge at 13,000rpm for 10 minutes to sediment debris, and the supernatant subsequently transferred into new micro-centrifuge tubes. Next, an equal volume of cold isopropanol (i.e. 400μl) was added to the obtained supernatant and left on ice for 20 minutes to facilitate DNA precipitation. The 1.5ml centrifuge tubes containing the supernatant were spun in a centrifuged again at 13,000rpm for 10 minutes, following which the supernatant was discarded and the tubes from the centrifuge left upside-down on a paper towel for 30 minutes in order to allow the isopropanol to evaporate. A volume of 150μl of TE buffer, comprising 10mM of Tris (tris(hydroxymethyl)aminomethane) and 1mM of EDTA (ethylenediaminetetra-acetic acid) (brought to pH+8 with HCl to down-regulate the activity of nucleases), was added to stabilize and protect the dissolving DNA from degradation. Only 1μl volume of the DNA sample was required for the polymerase chain reaction (PCR).

2.3.3 PCR for genotyping

To determine the genotype of *Igf2*-P0 KO mice, two separate PCR reactions were performed to identify the targeted P0 (i.e. the HSV-*tk-neo*^{r} cassette) and WT-P0 (i.e. the P0 promoter) forms of *Igf2*. Reaction mixtures were produced as per the protocol described in Table 2.2. The primer sequences for the *Igf2*-P0 gene promoter were obtained from Constância et al. (2000) and are listed in Table 2.3. The reactions were run in a Peltier Thermal Cycler (MJ Research, U.K.), using the programme described in Table 2.2. Once the reaction had finished 6μl of 6x DNA loading buffer was added to each reaction and a total of 15μl of the reaction/loading buffer mixture was loaded on a 1% agarose gel. Finally, 5μl of DNA ladder (Hyperladder IV, Bioline, U.K.) was loaded at both sides next to the reaction samples, and the gel subsequently run at 100V for 60 minutes, in order to allow the DNA to separate. Amplicons were visualized using a UV scanner. Identification of *Igf2*-P0 KO mice was accomplished by comparison of the resultant amplicons from each reaction. Thus, only *Igf2*-P0 KO mice will show an amplicon from reaction 1 (band at 740bp) as this PCR specifically identified the modification in *Igf2* that caused the P0 promoter to be ablated whereas all mice (KO and WT) will show an amplicon from reaction 2 (band at 495bp). Sincer there were noticeable (and obvious) size differences at birth,

between the growth retarded *Igf2*-Null KO mice and their WT-littermates, no PCR reaction were required for the identification of genotype, among mice of the *Igf2*-Null KO line.

Table 2.2: *The PCR reaction protocol.*

Mutant *Igf2*-P0 allele	**WT *Igf2*-P0 allele**	**Program-cycle**
14.6 µl of nuclease-free H_2O	14.6 µl of nuclease-free H_2O	1. 95°C for 15min
2.5 µl of dNTPs (2mM, Invitrogen, U.K.)	2.5 µl of dNTPs (2mM, Invitrogen, U.K.)	2. 95°C for 30sec
2.5 µl of 10x Taq buffer (Qiagen, U.K.)	2.5 µl of 10x Taq buffer (Qiagen, U.K.)	3. 62.2°C for 30sec
2 µl of $MgCl_2$ solution (25mM)	2 µl of $MgCl_2$ solution (25mM)	4. 72°C for 50sec
1 µl of primer WT forward (10µM)	1 µl of primer WT forward (10µM)	5. Goto step 2; 35 times
1 µl of primer *Igf2*-P0 reverse (10µM)	1 µl of primer WT reverse (10µM)	6. 4°C forever
1 µl of genomic DNA	1 µl of genomic DNA	7. End
0.4 µl of Taq Polymerase buffer (Qiagen, U.K.)	0.4 µl of Taq Polymerase buffer (Qiagen, U.K.)	
25 µl	**25 µl**	

Table 2.3: *The primer sets used for genotyping of the Igf2 gene.*

Primers	**Primer sequence**	**Genotype detection**
WT Forward	**5'-TCCTGTACCTCCTAACTACCAC-3'**	WT/*Igf2-P0*
WT Reverse	**5'-CAATCTGCTCCTGCCTG-3'**	WT
Igf2-P0 Reverse	**5'-GAGCCAGAAGCAAACT-3'**	*Igf2-P0*

2.4 Habituation to handling, test environment and procedures

2.4.1 Handling

Following weaning, at approximately four weeks of age, the mice were allowed to acclimatize to their new environment for at least a two week period, before being handled daily for two weeks (approximately 1 minute per subject per day). Body weight measurements were instigated during this initial handling regime.

2.4.2 Measurement of body weight

Body weights of all mice were recorded on a regular basis as an index of general health. Weights were registered at the same time of each day over two week period prior to behavioural testing (at around 17:00 hours, to coincide with the end of

the experimental sessions). However, during the period of behavioural testing, body weight measurements were reduced to a twice a week schedule.

2.4.3 Behavioural testing environment

Behavioural testing was carried out in custom-built sealed and air-conditioned testing rooms. Testing rooms were lit by fluorescent lights which could be dimmed to varying degrees. Although temperature and humidity levels were not strictly controlled as in the holding rooms, these parameters were generally maintained at around 21 ± 2°C and 50 ± 10% respectively. Rooms were thoroughly cleaned once a week.

2.4.4 Protocol for the water restriction schedule

Prior to testing in behavioural paradigms where liquid food reward 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), comprising 4-hour access to water per day for four days, followed by 2-hour water access per day thereafter throughout the duration of the testing (with schedule placed at the same time each day). Importantly, subjects losing >20% of their *ad libitum* body weight or showing clinical symptoms of dehydration were immediately given *ad libitum* water access until normal *ad libitum* body weight was reestablished. Subjects were allowed *ad libitum* access to standard laboratory chow, throughout the duration of the water deprivation schedule. Furthermore, in an effort to ensure the general health of the test subjects was not being compromised by the water restriction schedule, general health and body weight was monitored on a regular basis. Any mice showing signs of dehydration, malnutrition or illness were immediately reported to the unit named animal care welfare officer (NACWO) and, if necessary, to the veterinarian.

2.4.5 Reinforcer preference test

Once the body weight of mice had stabilized following regulated water consumption (generally after 10 days), subjects undergoing appetitively motivated behavioural tasks (i.e. 5-choice serial reaction time task and Stop-signal serial reaction time task), were habituated to the reinforcer (10% condensed milk solution, Nestle

Ltd, U.K.). Briefly, the reinforcer preference test was carried out in a number of holding cages (285 x 130 x 120mm) with a single subject per cage, during a single 10-minute session per day, across a six-day period. During the first two sessions, subjects were allowed to habituate to the test apparatus, while general water consumption was measured by placing two containers (max vol.= 3ml 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, whereas 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 (and thus, the total daily consumption), as well as the daily preference for the condensed milk. However, 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. Moreover, the data on total volume of liquid consumed was normalized for body weight differences using Kleiber's 0.75 mass exponent (Schmidt-Nielsen, 1990).

2.4.6 Examining behavioural phenotypes: experimental control measures

In order to ensure further consistency in data collection and to avoid influences of potential confounding variables, a number of precautionary measures were employed to provide greater experimental control. For instance, 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 minimize any behavioural variation due to temporal location of behavioural examination, where possible, individual subjects were tested at the same time each day. In addition, possible 'order of experimental run' effects were negated by running the *Igf2*-KO mice and the WT-littermate controls in a pseudo-random order. To minimize possible

confounds related to cage/litters, the experimental subjects were drawn from as large number of cages/litters as available (minimum of 10 litters/cages). Further to these measures, the data were routinely screened for any such effects. However, no data from the experiments reported in current thesis displayed any signs of systematic variations which could be attributed to the aforementioned factors.

2.4.7 The collective WT-control group: pooling of WT-littermates from different mutant lines

Marked differences were noted across control groups of WT littermates from the two separate control lines, in terms of body size, weight and somatic development (these data will be further elaborated upon in Chapter III). While these physical differences between the two WT-control groups is likely to stem from varying access in early postnatal nutrition (i.e. WT-Null controls potentially gain greater access to the maternal milk prior to weaning due to the significantly smaller body size of the *Igf2*-Null KO mice), it is uncertain whether these two groups of WT-littermate controls are equivalent in terms of cognitive/behavioural functions, and if not, which of the two groups of WT littermates provides a more reliable or valid baseline. Therefore, it was decided to pool the two WT-littermate groups together into a collective WT-control group. This might be particularly important since postnatal environmental factors (not of interest to the purpose of present study) such as postnatal nutrition or abnormal behaviour of KO littermates, might suppress potential differences between intrauterine growth retarded (IUGR) subjects and nonIUGR subjects due to placental insufficiency. In addition, pooling the WT-littermate groups together enables a more meaningful interpretation of the data, as well as improves the sensitivity of the analyses of variance; by increasing the *n* (i.e. sample size) of the control group and reducing the number of group-comparison required.

However, pooling of WT littermates into a single control group also introduces potential experimental confounds. For instance, the heterogeneity between the two groups of WT littermates *per se* imposes a serious issue of the legitimacy of pooling together WT littermates of different mutant lines into a single control group. Also, if mice from either group of WT littermates are abnormal due to some postnatal environmental factors, the mice of that control group might induce a behavioural

difference between *Igf2*-KO mice and WT controls, which is not due to placental insufficiency induced IUGR. In an effort to address these issues, each analyses of between-genotype comparison was followed-up by an independent T-test analysis and/or a repeated measures analysis on segregated WT-control groups (in order to exclude the possibility of genotype-related interactions), and any significant difference revealed between subjects of different group of genotype was further probed by a within-mutant line comparison (i.e. *Igf2*-P0 KO against WT-P0, and *Igf2*-Null KO against WT-Null). Results from each analysis of every experiment can be found in the appendices of the thesis and are cited when required.

2.5 Behavioural apparatus

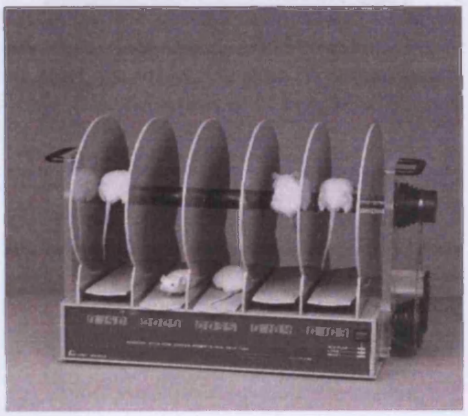
The current section describes the experimental apparatus employed in this thesis. However, the experimental procedures in relation to the apparatus are described in the subsequent chapters.

2.5.1 Locomotor activity chambers

Testing of locomotor activity (LMA) was carried out in an apparatus consisting of eight clear Perspex chambers (each 210 x 360 x 200mm, width x length x height), with embedded two infra-red beams crossing each cage 30mm from each end and 10mm from the floor of the chamber. Beam breaks were recorded as an index of activity, using a computer running custom written BBC Basic V6 programmes with additional interfacing by ARACHNID (Cambridge Cognition Ltd, Cambridge, U.K.). Data were stored as the total number of beam-breaks from each session, as well as the number of beam-breaks made over a 5-min time-blocks (as used in Humby, Laird, Davies and Wilkinson, 1999).

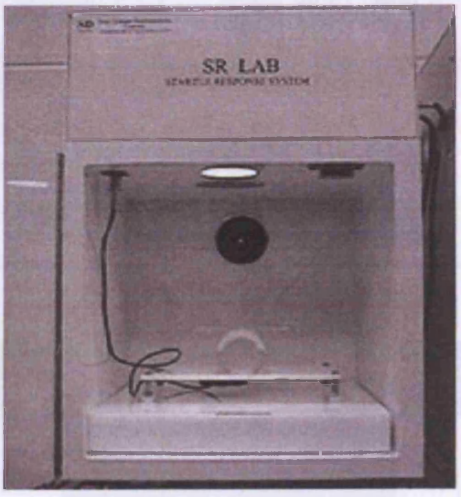
2.5.2 The Rotarod

The Rotarod (Ugo, Basile, Italy) apparatus consisted of a rotating bar (25mm diameter) and under the control of a system device that allowed for altered rotational speed. The rotational speed could be set in two modes: accelerated (5-50rpm) over a 5-min period, or held fixed at any speed (between 5-50rpm). The bar was divided into five segments, enabling testing of a single mouse within each of the segments. A lever beneath each segment of the bar was raised, initiating a timer. When a mouse fell from the bar, the lever was depressed, and the timer automatically stopped (as used in Carter et al., 1999). Thus, the latency in second and the rotational speed (rpm) at fall could be recorded.



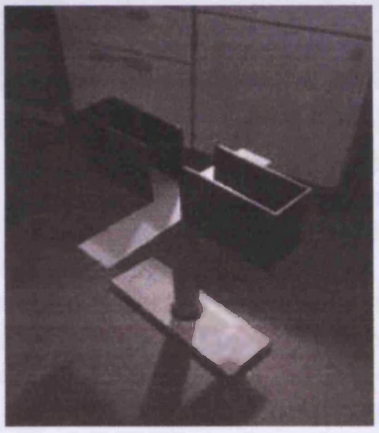
2.5.3 Startle chamber

The startle chamber apparatus consisted of a ventilated and soundproofed SR-LAB startle chamber (San Diego Instruments, CA, USA) containing a non-restrictive Plexiglas cylinder (35mm in diameter) mounted, onto which a single mouse was placed. The Plexiglas cylinder was mounded on a Perspex plinth with a pressure sensitive piezoelectric accelerometer unit attached below the Plexiglas cylinder. The motor response of a subject to a white noise stimuli (generated from a speaker 120mm above the cylinder) were recorded via the piezoelectric accelerometer which converted flexion plinth vibration into electrical signals Responses were monitored for 50msec following the onset of the stimulus. A session consisted of exposure to 36 acoustic stimuli, presented with a 10-sec random interval, against a background noise of 70dB. Stimuli were presented at 120dB within each sessions; 4 no stimuli, 12 x pulse-alone, and 5 at each 2, 4, 8, 16dB prepulse amplitude. The duration of each prepulse and pulse stimuli was 0.3sec, with a 0.7-sec gap in between (as used in Davies et al., 2005).



2.5.4 Elevated plus maze

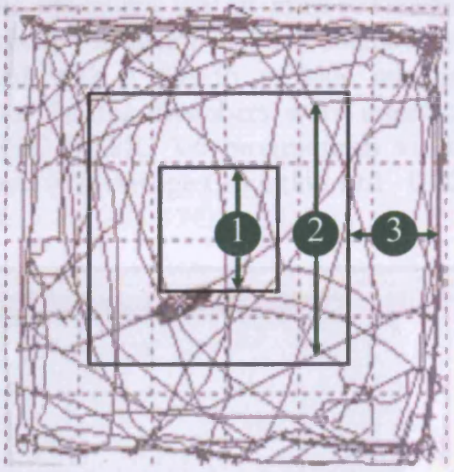
The plus maze was constructed of dulled black Perspex and consisted of two exposed open arms (175 x 78mm, length x width) and two enclosed arms (190 x 80 x 150mm, length x width x height) with an open roof. Equivalent arms were arranged opposite one another. The plus maze was positioned 300mm above the floor and illuminated evenly at 15 lux (mixture of low level white and red light), as used in Davies et al., 2005.



2.5.5 Open field arena

The open field apparatus consisted of a square-shaped arena (750 x 750 mm, length x width), constructed of white plastic and illuminated evenly at 15 lux; mixture of low level white and red light (as used in Davies et al., 2005)

1 inner-square (200 x 200mm)
2 middle-square (400 x 400mm)
3 peripheral region (100mm, width)

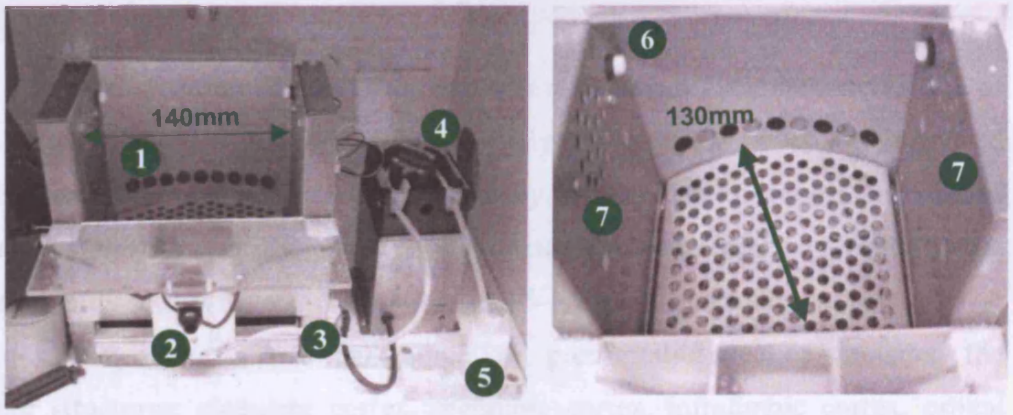


2.5.6 Free-exploratory apparatus

The free-exploratory apparatus consisted of two compartments constructed of either white or black Perspex (each of 300 x 300 x 300mm, width x length x height). The two compartments, separated by a wall, were connected by a small square opening of 70 x 70mm. Coarse grained sandpaper was placed pseudo-randomly on the floor on one of the compartments to enhance tactile discriminability between the two compartments (as used in Plagge et al., 2005).

2.5.7 Apparatus for the 5-choice serial reaction time task (5-CSRTT) and the Stop-signal serial reaction time task (SSRTT)

Behavioural analysis was performed in 9-aperture operant chambers based upon a design developed for use in rats and modified for use in mice in collaboration with Cambridge Cognition Ltd., U.K. In short, these chambers consisted of a food magazine and an array of 9 apertures (10mm diameter) set into the opposite curved wall (at a distance of 130mm). The magazine housed a small well in its base (to which reinforcer was delivered via a peristaltic pump in 0.8mm silicone tubing) and a 2.5Watt tray light. The magazine could be accessed from the main chamber through a clear Perspex panel hinged at the top; the raising of this panel was recorded via micro-switch. Behind each aperture was a 2.5Watt light, which brightness could be altered through a series of resistors, and the entrance to each aperture was spanned by a vertical infra-red beam. For the 5-CSRTT settings, four of the nine apertures were covered with opaque plastic film (apertures 2, 4, 6 and 8 as counted from the left), whereas only apertures 2 and 7 were uncovered for the SSRTT settings. Moreover, two loudspeakers, two 2.5Watt house lights and two infra-red LEDs were set into the side walls of the chamber; the latter provided background illumination of the chambers and facilitated viewing of the mice in the chambers via an infra-red camera (Watac WM6, Tracksys Ltd., U.K.) located 100mm above the chamber. Each chamber was enclosed within a soundproofed wooden box fitted with a fan to provide adequate ventilation and a constant level of background noise. Testing chambers were interfaced with an Acorn RISC-PC running custom written BBC BASIC V6 programmes via the ARACHNID system and 9-aperture box interface panels (Cambridge Cognition Ltd., U.K.), as described in Humby, Wilkinson and Dawson, 2005.



1 response array (9 apertures, 10mm 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 reservoir (10% condensed milk solution), **6** house light and **7** loudspeakers. Note that in the right hand photograph, four of the nine response apertures are blocked (2, 4, 6 and 8 as counted from the left), as in the 5-CSRTT settings.

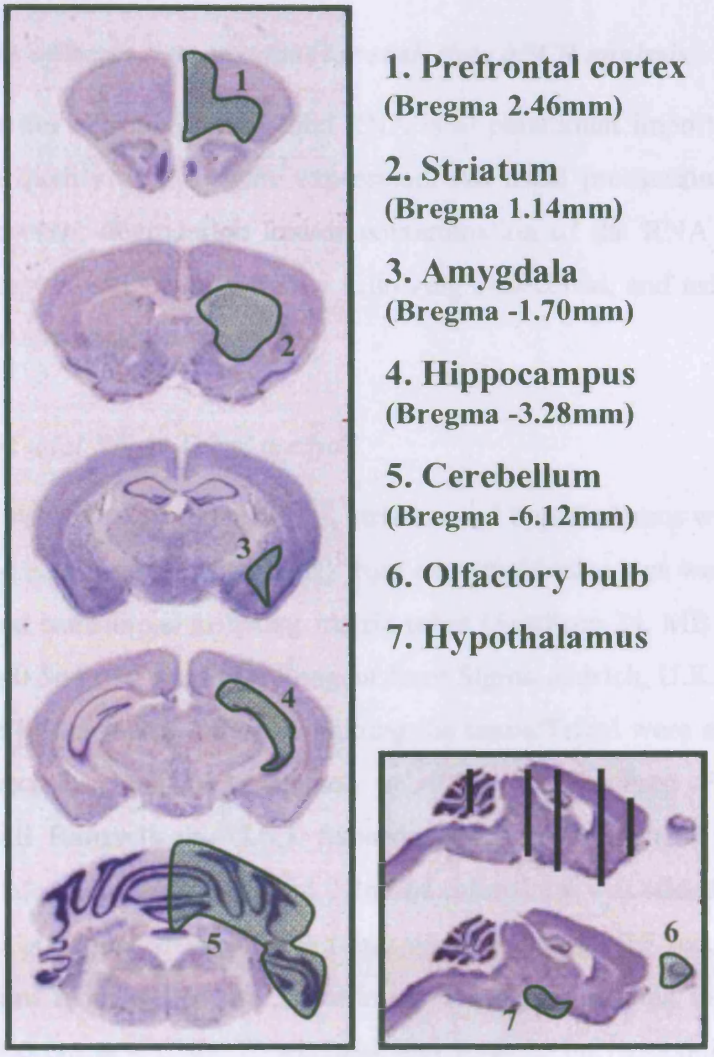
2.6 Culling protocol

At the end of the experiment, or in case of illness/injury, subjects were culled through cervical dislocation.

2.7 Brain dissection

Cerebral tissue used for neurochemical analyses was collected from behaviourally naïve mice (aged 6-8 months) and was dissected immediately following removal. Dissection was performed with a razor blade (single blade per brain) on metal plates pre-cooled with wet ice under the plates, as this protocol limited neurochemical and structural degradation during dissection, and facilitated the dissecting procedure by making the tissue more amenable to cutting. In order to ensure consistency of dissection across animals, a standardized dissection procedure was employed. Thus, six brain regions were dissected from the left hemisphere of the brain (right hemisphere was frozen intact). These regions were the following; olfactory bulb, the prefrontal cortex, the striatum, hypothalamus, hippocampus, amygdala and cerebellum (see Figure 2.3). Although the protocol was based on estimation of a brain of 11mm in length (excluding the olfactory bulbs), appropriate adaptations to the procedure were made accordingly for brains deviating from this standard.

The measurements detailed below represent distances from the anterior end of the brain (excluding the olfactory bulbs). Initially, the olfactory bulbs were removed from the intact brain by dissecting off vertically at the nexus of the olfactory bulbs and the frontal cortex. Next, the striatum, including the medial and orbitofrontal cortices, was dissected from a coronal slice (0.5-1.25mm, in thickness), just anterior to the forceps minor corpus callosum. The prefrontal dissection included the following structures; cingulate cortex, prelimbic cortex, infralimbic cortex, orbital cortex and agranular insular cortex. The striatal dissection was obtained from a coronal slice (2-3mm), where the anterior commisure and the lateral ventricles were used as landmarks. The striatum dissection included the caudate putamen, and nucleus accumbens. The amygdala was dissected from a coronal section (4.5-5.5mm), and was defined as the triangular-shaped region bordered by the corpus callosum. Hence, this dissection consisted of the basolateral and lateral amygdala nuclei.



1. Prefrontal cortex
(Bregma 2.46mm)

2. Striatum
(Bregma 1.14mm)

3. Amygdala
(Bregma -1.70mm)

4. Hippocampus
(Bregma -3.28mm)

5. Cerebellum
(Bregma -6.12mm)

6. Olfactory bulb

7. Hypothalamus

Figure 2.3: *A schematic view of the key cerebral regions dissected for future use in present research. Regions 1-5 are displayed from a coronal view, whereas regions 6-7 are viewed from a sagittal perspective The brain atlas pictures were obtained from; http:www.Brainmaps.org.*

The hippocampal dissection was made from a coronal section (6-7mm), and obtained with relative ease due to the distinct formation of the hippocampus, although care was taken to ensure that overlying cortical tissue was removed. The hippocampal structure consisted of both ventral and dorsal regions. Similarly, the whole cerebellum was also easily removed due to the external location and ease of identification. Finally, the dissected cerebral tissue was frozen on dry ice, placed into a sterile container and stored at -80°C for future analysis.

2.8 Real-time qPCR methods

2.8.1 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 data of gene expression. 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 dissections, and using RNA-free working surfaces and consumables.

2.8.2 Isolation of total RNA: Trizol method

Cerebral tissue (only hippocampus, striatum and hypothalamus were processed for the current research, see Chapter VIII) from extracted brains was weighed using a microbalance, and transferred to lysing matrix tubes (FastPrep-24, MB Biomedicals, U.S.) containing 0.5ml of Trizol (Tri Reagent from Sigma-Aldrich, U.K.) per gram of tissue. Next, the lysing matrix tubes containing the tissue/Trizol were shaken for 15, and then 10 seconds, and homogenised at 4°C in a FastPrep FP120 micro-homogenizer (MB Biomedicals, U.S.). Subsequently, the supernatant was removed from the matrix tubes and discarded, and 0.2ml of chloroform was added for each 1ml of Trizol (added previously). Again, the tubes were shaken for 15 seconds and then incubated at room temperature for 2-3 minutes. The mixture was then spun in a centrifuge at 4000rpm at 4°C for 15 minutes, and supernatant (containing the RNA) was transferred to a fresh screw cap polypropylene centrifuge tube. A volume of 0.5ml of isopropanol was next added per 1ml of Trizol, and samples were then incubated at room temperature for 10 minutes. The mixture was then spun in a centrifuge at 4000rpm at 4°C for 10 minutes, forming a gelatinous pellet, after which the supernatant was removed and discarded. Subsequently, the RNA pellet was washed in 1ml of 75% ethanol per 1ml of Trizol in the intial preparation, vortexed and spun in a centrifuge at 4000rpm at 2-8°C for five 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.

2.8.3 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 260nm, the ratio of absorbance at 260nm and 280nm (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/microlitre, 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 is normalised to a 1 cm path. The generally accepted extinction coefficient for RNA is 40.

The spectrophotometer enabled highly accurate analyses of particularly small samples. Surface tension was used to hold a column of liquid sample in place while a measurement was made. A small quantity of the sample (1-2 μl) 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 generally carried out within 10 seconds, while the spectrum and the analyses were displayed on a screen and archived, of an attached PC computer. Once a measurement was completed, the sample was wiped from the measurement pedestals.

2.8.4 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 100μl with RNase free water, and 300μl of RLT buffer was added to the sample and mixed well. Subsequently, 250μl of absolute ethanol was added to the RNA/RLT solution and mixed thoroughly, and then 700μl 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 10,000rpm, applied over to a mini-spin column membrane of the collection tube, and spun again for 15 seconds at 10,000rpm. Once, the RNA had been transferred into a

new collection tube, 500μl of buffer RPE was added and the solution then spun in a centrifuge for 15 seconds at 10,000rpm 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 two minutes at 13,000rpm to dry the RNeasy membrane. Next, the RNA spin column was placed in a new 2ml collection tube and spun in a centrifuge for one minute. The column was then transferred into a new 1.5 collection tube and 20μl of RNase-free water was pipetted directly onto the membrane and allowed to stand for one minute, after which it was spun in a centrifuge for a minute at 10,000rpm. This step was repeated but with only 10μl of RNase-free water this time. RNA was eluted in a total volume of 30μl nuclease-free water and again was quantified by absorbance using the NanoDrop® ND-1000 UV-Vis Spectrophotometer machine.

2.8.5 cDNA synthesis

The synthesis of first-strand cDNA from the total extracted RNA was carried out using Sprint^{TM} RT Complete Products Kit (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 2.5μg of total RNA in a final volume of 20μl, based upon the previous determination of total RNA concentration. Next, the total RNA/H_20 samples were pipetted into individual wells of the Sprint RT complete product and mixed thoroughly by pipetting up and down. The wells containing the RNA/H_20 reactions 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.

2.8.6 Quantitative PCR primer sets

The primer sets used for the real-time quantitative PCR analysis, were designed using customised software provided by the Babraham Institute Bioinformatic department, of the Babraham Institute, Cambridge, UK. Each of the primer sets was designed across intron-exon boundaries, thus eliminating signals from contaminating genomic DNA along with the working concentrations that were optimised to minimise the possibility of primer-dimer formation (Table 2.4).

Table 2.4: *List of qPCR primer sets used for the gene expression analysis described in Chapter VII.*

Gene	Primer	Primer sequence	Concentration
5HT-1A	Forward	5'-GACGGTCAAGAAGGTGGAAA-3'	700nM
	Reverse	5'-CACTGCGCCTGCAGTCCC-3'	700nM
5HT-2A	Forward	5'-GGTTTCCTTGTCATGCCCG-3'	300nM
	Reverse	5'-GACGCCGTGGAGAAGAGC-3'	300nM
5HT-2C (long)	Forward	5'-CATCATGAAGATTGCCATCGTT-3'	700nM
	Reverse	5'-CGCAGGTAGTATTATTCACGAACACT-3'	700nM
5HT-2C (short))	Forward	5'-ATCGCTGGACCGGAGTTTC-3'	300nM
	Reverse	5'-GGGTCATTGAGCACGCAGG-3'	50nM
Gaba-α2	Forward	5'-AGTCAGGTTGGTGCTGGCTAA-3'	300nM
	Reverse	5'-CCGATTATCATAACCATCCAGAAGT-3'	300nM
Gaba-α3	Forward	5'-CTTCTTCAACTCCAACAGCGATT-3'	700nM
	Reverse	5'-TGTTGTAGGTCTTGGTCTCAGCA-3'	700nM
18S	Forward	5'-GTAACCCGTTGAACCCCATT-3'	300nM
18S	Reverse	5'-CCATCCAATCGGTAGTAGCG-3'	300nM

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 cDNA 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 *C*t values (see section 2.8.8 below) of the NTC reactions were compared to the *C*t values obtained in the DNA-containing PCR reactions with the same primer combinations and those showing the greatest difference between the NTC sample and the DNA sample were deemed as the most optimal combination of primer pairs for future work (see Table 2.4).

2.8.7 Real-time quantitative PCR

The study of gene expression 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 were set up in accordance with the protocol listed in Table 2.5, where the purpose of stage six and seven in the programme is to obtain "melting curve" data, which enabled confirmation

that only a single type of amplicon was produced, and that no primer dimerization had occurred.

Table 2.5: *List of qPCR primer sets used for the gene expression analysis described in Chapter VII.*

Reactions	Program
1.4 µl of H_2O	1. 95°C for 10'
7.5 SensiMix 2X	2. 57°C for 0:15
0.3 Syber (50X)	3. 72 °C for 0:20
Forward primer	4. Goto Step 1; 40 times
Reverse primer	5. Ramp from 72°C to 95°C rising 1°C each step (melting curve analysis)
10 µl of cDNA (1/10)	6. Wait for 90'' of pre-melt conditioning on first step and for 5'' for each step afterwards.
	7. End
21 µl	

2.8.8 Real-time qPCR analysis: 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 (see Simon, 2003). 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).

2.8.8.1 Selection of internal control

In the present study, the RNA samples were derived from dissected cerebral tissue of *Igf2*-P0 KO mice and their relative WT-littermate controls (see section 2.7). However, due to the fact that samples (i.e. the cerebral tissues) varied in size, false expression data could be produced by the varying concentration of extracted RNA (or the converted cDNA) from each subject. Therefore, in order to normalise the qPCR reactions for the amount of RNA added to the reverse transcription reaction, an internal gene (otherwise known as a house-keeping gene) was required to establish a control. Importantly, the expression of the house-keeping gene needs to be abundant and unaffected by genetic abnormalities or chemical treatments (Thellin et al., 1999).

For these reasons, the 18S gene (rRNA) was selected as suitable control for the real-time qPCR analysis (see Thellin et al., 1999).

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

The change in expression of the target gene, after normalization to the house-keeping 18S rRNA, was assayed in two groups of samples; eight *Igf2*-P0 mutants and eight wild-type controls. Real-time qPCR was performed on each of the 16 samples of cDNA, with each sample run in triplicates (in effort to minimise sampling errors). The *C*t 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, Vrana and Freeman, 2008). The *C*t values for the 18S and the target gene mRNAs were averaged across the triplicates for each sample, prior to performance of the Δ*C*t calculation. The Δ*C*t value was calculated by subtracting average *C*t values of the target genes from the average *C*t values of the 18S (i.e. the control sample). Next, the subtraction of the Δ*C*t values of the control samples from the Δ*C*t values of the target gene samples, yielded the ΔΔ*C*t values. The negative values of this subtractions, the -ΔΔ*C*t values, were then used as the exponent of 2, and represented in "corrected" number of cycles to threshold (see VanGuilder et al., 2008, Livak and Schmittgen, 2001 and Chapter VII for further details), 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.

2.8.8.3 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 *C*t value. The *C*t value was derived from a log-linear plot of the PCR signal against the cycle number, which depicts the *C*t value as an exponential instead of a linear term. Hence, the data was converted to a linear form using $2^{-\Delta Ct}$ (transformed data). In order to examine the statistical significance of the relative change, independent T-tests were performed on the transformed data.

2.9 General data presentation and statistical methods

The data were 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 16.0 for windows (LEAD Technologies Inc., Apache Software Foundation, U.S.). The data were screened for skewed variance, kurtosis and outliers (<2.5 standard deviation) prior to statistical analysis, and logarithmic (by power of 10) or arcsine transformations were applied on skewed data (percentage data in particular) in an effort to assure normal distribution. The data were analysed by either, one-sample, independent sample or paired sample Student's T-test, one-way analysis of variance (ANOVA) or repeated measures analysis of variance (ANOVA), where appropriate. *Post hoc* pairwise comparisons were performed using Tukey's HSD test, with alpha values of <0.05 regarded as significant. Furthermore, a one-way Kruskal-Wallis test was conducted on the data (Monte Carlo exact test of 0.99% confidence level, used as it is more appropriate for larger sample sizes), if or when assumptions of normality or homogeneity (Levene's test) of variance were violated. Any significance in mean difference noted by the Kruskal-Wallis test was followed up by a Mann-Whitney test and a Bonferroni correction, for a *post hoc* comparison. Greenhouse-Geisser degrees of freedom (df) corrections were applied as necessary to repeated-measures factors in relevant ANOVAs (nominal values for the relevant degrees of freedom are reported), and Wilcoxon signed-rank test was applied as a nonparametric substitute for the repeated measures analysis where or if required. Moreover, in terms of the gene expression data described in Chapter VII, a *Benjamini Hochberg* false discovery rate correction testing (Benjamini and Hochberg, 1995) was used to adjust *p*-values for false positives, due to multiple testing. See individual chapters for further details.

Chapter III

Initial Developmental Characterization and Behavioural Phenotyping of Mice from the Igf2-P0 and Igf2-Null Knock-out Lines

3.1 Introduction

The *Igf2* gene which spans 30 kb, and comprises nine exons and four promoter regions (designated P0-P3, O'Dell and Day, 1998) was one of the first genes shown to be imprinted (DeChiara et al., 1990). *Igf2* lies on the distal region of mouse chromosome 7 (corresponding to 11p15 in humans, *IGF2*) and encodes the 67 amino acid monomeric plasma protein IGF-II (displays 47% animo acid sequence homology to insulin), which is known to play a key role in both foetal and placental development (Constância et al., 2002; DeChiara et al., 1990, and Chapter I, section 1.4.1 for further details). *Igf2* promotes growth via paracrine and/or autocrine actions, which stimulate cell proliferation, differentiation and survival (O'Dell and Day, 1998). Expression of *Igf2* becomes most widespread in foetal and placental tissue during mid to late gestation, and is more abundant than *Igf1* gene expression, in both rodents and humans (Fowden, 2003). In fact, plasma concentrations of IGF-II are 3-10 fold higher than those of IGF-I during late gestation, but expression of *Igf2* declines dramatically following birth, and in rodents the IGF-II content disappears from most tissues except the brain (i.e. choroid plexus, leptomeninges and potentially in the developing retina) by weaning (Chao and D'Amore, 2008). As a consequence, IGF-II is almost abolished in adult plasma. However, *IGF2* expression is apparent during adulthood in humans, with transcripts deriving from an adult-specific promoter (de Pagter-Holthuizen et al., 1987).

As mentioned previously in Chapter I (section 1.6), there are several different transcripts of the *Igf2* gene in the mouse, expressed from the *Igf2* promoters P0-P3, each encoding the mature IGF-II polypeptide (Constância et al., 2005). The promoters P1-P3 are expressed in mesodermal, endodermal, extraembryonic tissues, whereas the P0 promoter is solely expressed in the labyrinthine trophoblast cell-layers of the placenta (Constância et al., 2002). Two mouse models have been generated using deletion of cell-type specific promoters to separately evaluate the roles of *Igf2* in

foetal and placental development. In mice where the labyrinthine trophoblast-specific *Igf2*-P0 transcript is exclusively abated (i.e. the *Igf2*-P0 KO model), there is a marked reduction in placental *Igf2* mRNA, although the foetal circulation of IGF-II is unaltered. In the *Igf2*-P0 knockout (KO) mice, overall placental weight is also reduced compared to wild-type (WT) from embryonic (E) day 12; however this reduction occurs across all placental layers proportionately, suggesting that IGF-II dependent growth of the labyrinthine exchange surface may regulate growth of the placenta as a whole (Constância et al., 2005). Nevertheless, foetal growth in the mice remains at a normal growth rate until E16, but declines subsequently and results in intrauterine growth restriction (IUGR), indexed by a birth weight of average 78% that of WT littermates (Constância et al., 2002). The passive permeability of the placenta of *Igf2*-P0 KO mice is also reduced at E19, due to decreased labyrinthine surface area and increased thickness of the exchange barrier.

In comparison, complete ablation of *Igf2* transcripts from all four foetal and placental promoters (P0-P3) induced in the *Igf2*-Null KO mouse model, results in concurrent foetal and placental growth deficiency around mid gestation (Baker et al., 1993). Although the reduction in placental weight of the *Igf2*-Null mutant placenta is equivalent to that of *Igf2*-P0 up to E14, the placenta growth restriction becomes subsequently more severe, and at E16 and E19 the *Igf2*-Null KO placenta is 14-24% lighter in weight in comparison to *Igf2*-P0 placentas (Constância et al., 2005). Hence, it is likely that foetal-placental expression of *Igf2* from the P1-P3 promoters might be more involved in regulating placental growth after E14. In a similar vein, the foetal growth deficiency is also far more severe among *Igf2*-Null KO mice than in *Igf2*-P0 KO mouse model that continue to express *Igf2* in their foetal tissue, at only approximately 60% of WT weight at birth (DeChiara et al., 1990).

In recent years, there has been a considerable amount of research carried out on the function of the *Igf2* gene in foetal and placental development, particularly in terms of the regulatory involvement of *Igf2* in the balance between the placental nutrient supply via the System A amino acid transporter, and the endocrine signalling of foetal nutrient requirements (Dilworth et al., 2010; Constância et al., 2008 and 2005). In contrast, to date there has not been any data published on the long-term cognitive and behavioural consequences, when this key maternal/supply-foetal/demand interplay is compromised by attenuated *Igf2* expression. That is to say, there has not been any published effort made to examine emotional, cognitive, and

other behavioural functioning of mice from either *Igf2* KO lines. Furthermore, while much work has focused on both foetal and placental characterization of the *Igf2*-P0- and *Igf2*-Null KO mice during intrauterine life, and basic growth pattern during early postnatal life, no detailed assessments have been made on the development of early somatic morphology or long-term growth into adulthood. Therefore, the main objectives of the present chapter were twofold; firstly, to examine the effects of placental deficiency and foetal growth restriction on early body growth and somatic/neurological development among *Igf2*-KO mice relative to WT-littermate controls, and secondly to conduct a preliminary characterization of the *Igf2* KO mice, in terms of basic motoric, sensory and emotional aspects of behaviour.

To address the first question, body weight was recorded at birth and monitored on alternate days across the first 100 days of postnatal life, along with several somatic measurements. The somatic indices of early postnatal development were registered, included the unfolding of the ear pinna, incisor eruption, eye opening, and fur growth. To address the second question, behavioural analyses were carried out across a number of test batteries. Basic locomotion was assayed using locomotor activity (LMA) chambers equipped with infrared motion-detectors, whilst rotarod apparatus was employed to further examine locomotor ability, along with motor learning, balance, and motor coordination. An assessment of emotional reactivity of the mice was carried out with a simple responsive test to a novel foodstuff and the response and habituation to acoustic stimuli that induce a startle response (Li, Du, Li, Wu and Wu, 2009). In combination with this latter assessment, prepulse inhibition (PPI), where the presence of a moderate stimulus prior to an exacerbated startle inducing stimulus attenuates the overall response to the startle stimulus will also be measured. The startle-PPI paradigm provides a valuable index of sensory-motor gating and reflex which have frequently been reported to be impaired in neuropsychological disorders such as; schizophrenia (Geyer, Krebs-Thomson and Swerdlov, 2001), attention deficit hyperactivity disorder (ADHD, Feifel, Minassian and Perry, 2008) and anxiety disorders (Duley, Hillman, Coombes and Janelle, 2007). The findings from this initial series of experiments will provide a developmental and behavioural profile of mice from both *Igf2* KO lines (i.e. the *Igf2*-P0 and *Igf2*-Null KO) and at the same time indicate any potential experimental confounds for future behavioural studies.

3.2 Materials and Methods

Full methodological descriptions can be found in the relevant sub-sections of the General Methods Chapter (Chapter II).

3.2.1 Subjects and animal husbandry

A subset of mice from the total litter cohort, consisting of mice from the *Igf2*-P0 and *Igf2*-Null KO lines, and WT-littermate controls (WT-P0 and WT-Null mice), were weighed and examined on several measures of somatic development during the early days of postnatal life (see Table 3.1). Behavioural testing was also carried out on a subset of mice, at the age of 3-6 months. The sample size (*n*) for each subset of mice that were examined on somatic indices and individual behavioural tests is listed in Table 3.1. General housing, handling and behavioural testing conditions have been detailed previously in Chapter II, section 2.1.

Table 3.1: *The sample size and genotype of each cohort-subset of mice assayed on measurements of somatic development, and behavioural tests.*

Behavioural Apparatus	Genotype and sample size (*n*)		
	Igf2-P0	*Igf2*-Null	WT (WT-P0/WT-Null)
Weight/developmental measurements	40	20	78 (39/39)
Locomotor activity	17	8	32 (18/14)
Rotarod	19	9	37 (19/18)
Acoustic startle and pre-pulse inhibition	21	15	48 (37/11)
Reactivity to novel food-substance	36	20	67 (37/30)

3.2.2 Physiological measures

3.2.2.1 Assessment of birth weight and somatic indices of development

Body weight was registered on the day of birth, and subsequently monitored every alternate day for the first 100 days of postnatal life. The somatic indices of development were systematically examined by visual inspection on a daily basis until weaning, and consisted of the following parameters; the day of ear opening (the

unfolding of the ear pinna in both ears), incisor eruption (eruption of the upper incisors), fur growth (the appearance of complete coat of dark hair, including the abdomen) and bilateral eye opening (visible break in eye lids and membranes covering the eyes).

3.2.3 Behavioural measures

3.2.3.1 Locomotor performance

Spontaneous locomotor activity was tested in a battery of eight clear Perspex chambers, each of 210 x 360 x 200mm (see Chapter II, section 2.5.1 for further details), each equipped with two infrared sensors projecting across each cage at 30mm from each end and 10mm from the cage floor. The total number of beam-breaks made within a five-minute period (block) was recorded for a two hour session (thus total of 24 blocks), using a computer running custom written BBC BASIC V6 programmes with additional interfacing by ARACHNID. Short range movements of the subjects were registered as the total number of infrared beam-breaks, whereas runs through the entire chamber were indicated by successive beam-breaks at the opposite end of the chambers. Cages were thoroughly cleaned with 2% acetic acid between subjects. All animals were run in the dark between the hours 08:00 and 17:00 on three consecutive days in order to assess reactivity to novelty (indexed by the any change in performance upon repeated exposure to the test apparatus), with each animal run at a similar time on each day.

3.2.3.2 Balance and motor coordination

Mice were tested on the Rotarod apparatus (as previously described in Chapter II, section 2.5.2) in order to provide a more sensitive assessment of various aspects of motor function, such as; motor learning, balance and coordination. Subjects were tested on the rotarod across five consecutive trials, during which subjects were placed on the rotating rod of the apparatus which steadily accelerated in velocity from 5-50rpm throughout the entire session. The latency of subjects to fall from the rod was registered by the automatic system, as well as the velocity level of the rod-rotation at the time of fall. Each trial lasted for maximum of 4 minutes. An inter-trial interval of at least 30 minutes was provided to allow for recuperation. Following five consecutive trials, the performance of subjects on the rotarod apparatus was tested further over

two trials of 60 seconds each (with a 2-minute inter-trial interval) where the rotarod velocity was fixed at 35 and 45rpm. This further assessed balance and motor coordination while controlling for possible differences in stamina. The values for the five training sessions, latency to fall and rotational speed were averaged to produce single scores for each subject. Similarly, the latencies to fall for each of the sessions in the fixed configuration were also averaged together.

3.2.3.3 Acoustic startle and prepulse inhibition

In order to assay the integrity of sensory-motor circuitry, sensory-motor gating and gross auditory capability, mice were tested for acoustic startle and prepulse inhibition (PPI, see Chapter II, section 2.3.3 for apparatus description). Subjects were acclimatised to the startle chamber over a five minute period prior to testing, with a background white noise of 70dB. Subsequently, mice were subjected to three pulses of white noise at 120dB, followed by five blocks of six trials; each block consisting of two pulse-alone trials (120dB), and four trials in which a prepulse of 0.3 seconds duration was presented (pulse + prepulse trial) 0.7 seconds prior to the onset of the startle stimulus (one of each pre-pulse volume level of 2, 4, 8 or 16dB above background, presented pseudo-randomly). Additionally, four consecutive readings were taken without a stimulus presentation in order to account for movement unrelated to the startle response.

The data were corrected for locomotion in the absence of a stimulus by deduction of the average startle response at the four no-pulse trials from each pulse-alone or pulse+prepulse trial. Furthermore, as the response to a startle pulse was determined by the piezoelectric effect of the flexion at the base of the animal enclosure, all startle responses were normalised to body weight using Kleiber's 0.75 mass exponent (Schmidt-Nielsen, 1990). The startle responses to pulse-alone trials were calculated by averaging the responses to stimuli presentations 3-12, whilst the first two presentations were analyzed separately to provide measures of the stimulus response to a novel stimulus. The four presentations of each pulse+prepulse trials combination (at 2, 4, 8, 16dB prepulse amplitude above background) were averaged together and the following calculation was used to determine the prepulse inhibition (PPI):

$$\% \text{ prepulse inhibition} = 100 \times \frac{\text{(pulse-alone trial} - \text{pulse+prepulse trial)}}{\text{(pulse-alone trial)}}$$

3.2.3.4 Assessment of reactivity to a novel foodstuff

The reactivity to a novel foodstuff was assessed in mice following a period of 24-hour water restriction as described previously (see Chapter II, section 2.4.4), using a 10% condensed milk solution. The test of food neophobia (a choice between water and the 10% condensed milk solution) was carried out within the reinforcer preference/habituation testing, which was implemented prior to conditioned behavioural paradigms. Moreover, the liquid consumption data were adjusted for body weight differences, using Kleiber's 0.75 mass exponent. Further details of this procedure are described in Chapter II, section 2.4.5.

3.2.4 Statistical analysis

The data were subjected to either one-way analysis of variance (ANOVA) for a genotype (between-subject) comparison of performance for the entire experimental session, or a two-way repeated measures ANOVA to examine the effect of genotype on either performance of subjects across time (i.e. experimental blocks or days) or between different levels of the behavioural tests (such as concentration levels of natural rewards during preference tests), as well as possible interactions. Any interactions observed, were followed-up by an additional one-way ANOVA or repeated measures ANOVA between the individual levels of the within-subject factor, if or where required. Finally, data from the separate WT-lines were pooled together for analysis in order to increase the statistical power and for reasons mentioned previously (in Chapter II, section 2.4.7). However, every analysis of physiological and behavioural parameters was carried out separately by T-test between the WT-P0 and WT-Null mice, to ensure behavioural homogeneity between the WT-lines. The results of these analyses are presented in Appendix I.

3.3 Results

3.3.1 Physiological results

3.3.1.1 Neonatal health

The genotype of all mice was determined by standard PCR analysis (see Chapter II, section 2.3), along with registered birth weight. All of the subjects were monitored continuously throughout the duration of their lives for general health and wellbeing, by researchers and animal technicians. No indications of observable health problems were noticed in the *Igf2*-P0 and *Igf2*-Null gene KO mice. There were some incidents of neonatal mortality in the earliest days of postnatal life following birth (i.e. first 1-3 days), but these cases were few. Furthermore, since the *Igf2* gene mutation is inherited to offspring in a Mendelian manner, it is likely that the notably fewer births of *Igf2*-Null KO mice relative to their WT littermates reflect intrauterine mortality and re-absorption (although this may also have occurred in few cases with the *Igf2*-P0 KO mice). In addition, it is possible that maternal cannablisation of the smalles pubs could also occurred in some cases.

Table 3.2: *Number of mice born from each of the Igf2-KO lines.*

Mutation line	***Igf2*-P0 line**		***Igf2*-Null line**	
Genotype	**KO**	**WT**	**KO**	**WT**
Number of births	58	65	30	56

All of the mice displayed some degree of stress when initially handled, such as; vocalisation, faecal deposition and urination, although there was no discernible difference between the genotypes with regard to these behaviours. Moreover, the majority of mice became accustomed to handling after few days and did not exhibit any further observable signs of distress. No obvious difference in habituation to handling was observed across genotypes.

3.3.1.2 Early postnatal body weight and somatic indices of development

Mice of both *Igf2*-KO lines had significantly lower birth weights, than the WT-littermate controls (one-way ANOVA; $F_{2,135}= 147.76$, $p< 0.001$). The *Igf2*-P0 KO mice exhibited a ~25% reduction in birth weight relative to that of WT controls (*post*

hoc test; $F_{1,116}$= 91.78, p< 0.001), whereas the *Igf2*-Null KO mice were growth restricted by ~45% (*post hoc* test; $F_{1,96}$= 265.40, p< 0.001). The notable difference in growth retardation between the *Igf2*-KO mice were also found to be statistically significant (*post hoc* test; $F_{1,58}$= 65.72, p< 0.001). The overall growth rate across the first 100 days of postnatal life, was in addition, found to be greater among mice of the *Igf2*-KO lines than of WT controls (Figure 3.1A, onew-way ANOVA; $F_{2,135}$= 18.45, p< 0.001). Dividing the first 100 days of growth into four equal phases for further analysis, showed that *Igf2*-P0 KO mice underwent a period of accelerated weight gain relative to WT controls and *Igf2*-Null KO mice, between postnatal days 25-50 (Figure 3.1B, between-subject test; $F_{1,135}$= 10.07, p< 0.001, *post hoc* tests; $F_{1,116}$=19.74, p< 0.001). However, a repeated measures comparison between days 51-75 and 76-100 showed no difference in growth rate between *Igf2*-P0 KO and the other groups of mice ($F_{1,135}$= 0.13, n.s.). In comparison, the *Igf2*-Null mice only displayed a significant growth spurt during the first 25 days of postnatal life (Figure 3.1B, between-subject effect; $F_{2,135}$= 6.65, p< 0.001, *post hoc* test; $F_{1,96}$= 13.19, p= 0.001), following which growth rate was equivalent to that of WT controls (repeated measures analysis; $F_{1,96}$= 3.39, n.s.).

By postnatal day 100, the body weight of *Igf2*-P0 KO mice was still significantly reduced in comparison to WT controls (one-way ANOVA; $F_{1,135}$= 182.63, p< 0.001, *post hoc* test: $F_{1,116}$=21.08, p< 0.001), but this difference reflected only a ~7% reduction compared to the ~25% difference at birth, suggesting a significant period of accelerated catch-up growth. The body weight of the *Igf2*-Null KO mice was still markedly lower than WT controls (*post hoc* test: $F_{1,96}$= 365.07, p< 0.001), a reduction of ~38%, comparable to the ~45% difference at birth, suggesting an absence of catch-up growth during these first 100 days of life. Body weights from older mice (~400 days old) showed similar proportions of weight differences between the genotype groups (data not shown).

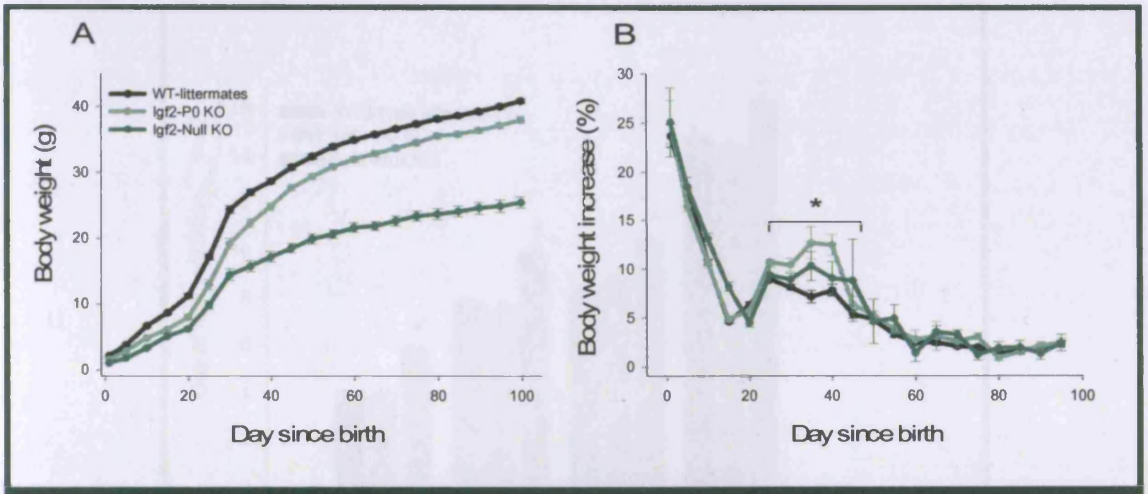


Figure 3.1: *Body weight over first 100 days of postnatal life.* ***(A)*** *Growth curves of early body weight over the first 100 days of postnatal life across mice of different genotypes.* ***(B)*** *Curves of early growth rate, in terms of percentage body weight increase, over first 100 days of postnatal life, across mice of different genotypes . Data are presented as mean values along with SEM.*

**Period of heightened growth rate in Igf2-P0 KO mice, significant at $p<0.05$.*

To gauge a measure of early somatic developmental, four physiological parameters were examined (Figure 3.2). No differences were noted for any of these somatic indices between the *Igf2*-P0 KO mice and the WT controls (ear opening (one-way ANOVA): $F_{2,135}= 57.23$, $p< 0.001$, *post hoc* test: $F_{1,116}= 0.15$, n.s., incisor eruption: $F_{2,135}= 26.80$, $p< 0.001$, *post hoc* test: $F_{1,116}= 0.26$, n.s., fur growth: $F_{2,135}= 24.04$, $p< 0.001$, *post hoc* test: $F_{1,116}= 2.93$, n.s., and eye opening: $F_{2,135}= 10.28$, $p< 0.001$, *post hoc* test: $F_{1,116}= 1.15$, n.s.). However, the *Igf2*-Null KO mice showed a significant delay in indices of early postnatal development of between 1-2 days, on all four of the somatic parameters (Figure 3.2); ear opening: $F_{2,135}= 57.23$, $p< 0.001$, incisor eruption: $F_{2,135}= 26.80$, $p< 0.001$, fur growth: $F_{2,135}= 24.04$, $p< 0.001$, and eye opening: $F_{2,135}= 10.30$, $p< 0.001$.

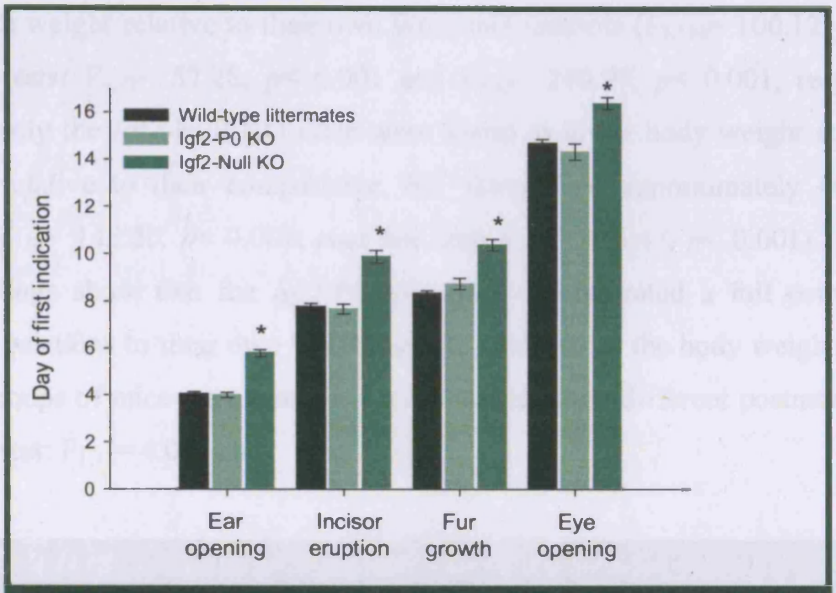


Figure 3.2: *Development of a number of somatic indices across mice of different genotypes. Data are presented as mean values along with SEM.*

**Significant at $p<0.05$.*

Analysis of early postnatal body weight, growth rate and somatic development was performed on the separate groups of the WT-littermate controls (i.e. WT-P0 and WT-Null controls, see Appendices 1.1 and 1.2). Although, body weight was found to be equivalent between the WT-P0 littermate controls and the WT-Null controls at birth, the WT-Null controls were found to have a notably higher body weight, by that of ~7%, at postnatal day 100 (WT-P0 (g): 39.35 ± 0.37, WT-Null: 42.40 ± 0.45). Thus, a differential postnatal growth pattern was noted over the first 100 days of life between the two WT-control groups where the WT-control mice of the *Igf2*-P0 mutant line showed an elevated growth rate between postnatal days 25-50 and the WT-Null control littermates displayed greater postnatal weight gain during the first 25 days of postnatal life (see Appendix 1.1). Due to these differences between the WT littermates of different control lines, it was important to carry out separate comparisons between the mice of each of the *Igf2*-KO lines with WT littermates from their own mutant line (see Table 3.2). However, these separate analyses did not reduce the pattern of effects reported previously; indeed they showed that *Igf2*-P0 KO mice achieved full catch-up to their WT controls and enhanced the difference reported for the *Igf2* KO mice. A one-way ANOVA, involving separate WT-littermate control group comparison, reported that both *Igf2*-P0 and *Igf2*-Null KO mice had significantly

lower birth weight relative to their own littermate-controls ($F_{3,134}$= 100.12, p< 0.001; *post hoc* tests: $F_{1,77}$= 57.28, p< 0.001 and $F_{1,57}$= 240.99, p< 0.001, respectfully), however only the *Igf2*-Null KO mice were found to lower body weight at postnatal day 100 relative to their comparative WT littermates (approximately 40% lower weight, $F_{3,134}$= 145.20, p< 0.001; *post hoc* test: $F_{1,57}$= 416.47, p< 0.001). Therefore, these findings show that the *Igf2*-P0 KO mice demonstrated a full compensatory growth in relations to their own WT-littermate controls, as the body weights between the two groups of mice were not found to be significantly different postnatal day 100 (*post hoc* test: $F_{1,77}$= 4.00, n.s.).

Table 3.3: *Birth and adult weight (day 100) between KO mice and WT controls of both Igf2 mutant lines. Data are presented as mean values along with SEM.*

Mutation line	***Igf2*-P0 line**		***Igf2*-Null line**	
Genotype	**KO**	**WT**	**KO**	**WT**
Birth weight (g)	1.60 ± 0.07	2.08 ± 0.02	0.96 ± 0.03	2.17 ± 0.04
Weigt at day 100 (g)	38.00 ± 0.54	39.35 ± 0.37	25.40 ± 0.86	42.39 ± 0.45

Furthermore, a separate analysis of somatic development between the two WT-control groups, revealed a significant delay in ear opening and incisor eruption among the littermates of the WT-Null control group, relative to the WT-P0 control groups (see Appendix 1.2). In addition, similar results to the between-genotype comparison described previously were obtained when the *Igf2*-KO mice were individually compared to their own WT-littermate controls, thus *Igf2*-P0 KO mice did not differ from their WT controls as before (ear unfolding: $F_{1,77}$= 0.72, n.s.; incisor eruption: $F_{1,77}$= 0.99, n.s.; fur growth: $F_{1,77}$= 2.17, n.s.; eye opening: $F_{1,77}$= 0.13, n.s., respectfully). However, the *Igf2*-Null KO mice showed greater latency in the unfolding of ear pinna (*Igf2*-Null KO: 5.80 ± 0.14 day, WT-Null: 4.23 ± 0.12 day, *post hoc* test: $F_{1,58}$= 72.13, p< 0.001), incisor eruption (*Igf2*-Null KO: 9.90 ± 0.26 day, WT-Null: 8.18 ± 0.17 day, $F_{1,58}$= 27.93, p< 0.001), fur growth (*Igf2*-Null KO: 10.40 ± 0.23 day, WT-Null: 8.36 ± 0.17 day, $F_{1,58}$= 39.62, p< 0.001) and bilateral eye opening (*Igf2*-Null KO (day): 16.40 ± 0.23 day, WT-Null: 14.92 ± 0.22 day, $F_{1,58}$= 9.95, p< 0.01) relative to their own littermate-controls.

3.3.2 Behavioural results

3.3.2.1 Locomotor performance

Locomotor activity performance was assessed with repeated measures analysis over three days, with separate 2-hour sessions each day. *Igf2*-P0 KO mice and the WT controls exhibited equivalent levels of locomotion throughout each session (between-subject test: $F_{1,47}= 3.17$, $p< 0.05$, *post hoc* test: $F_{1,38}$), although the level of locomotion was highest during the first session, but declined over the subsequent sessions (within-subject effect; $F_{2,2}= 10.$, $p< 0.001$) as the mice acclimatised to the activity chambers. In addition, ambulation was also noted be greatest at the beginning of each session but steadily reduced throughout the session blocks (see figure 3.3, Session 1 (within-subject test): $F_{7,403}= 50.05$, $p< 0.001$, Session 2: $F_{11,610}= 23.23$, $p< 0.001$, Session 3: $F_{10,566}= 14.53$, $p< 0.001$). In contrast, mice of the *Igf2*-Null KO line showed an overall reduction in locomotion across all three sessions in comparison to WT controls (Figure 3.4A, repeated-measures analysis (between-subject test): *post hoc* test: $F_{1,38}= 5.79$, $p< 0.05$).

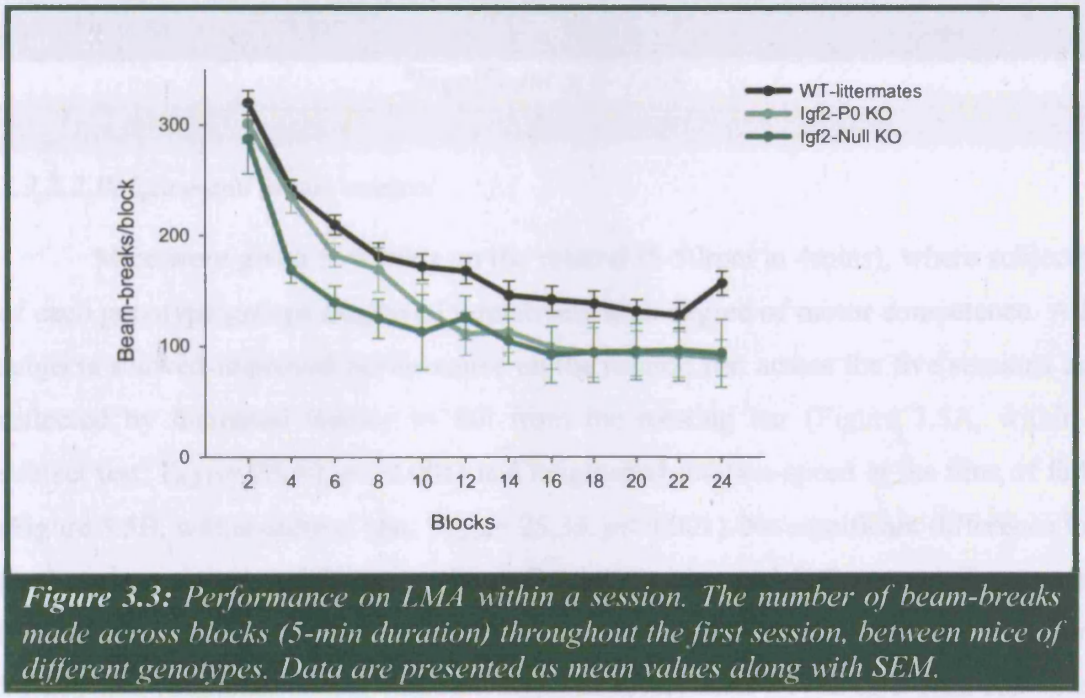


Figure 3.3: *Performance on LMA within a session. The number of beam-breaks made across blocks (5-min duration) throughout the first session, between mice of different genotypes. Data are presented as mean values along with SEM.*

The number of consecutive beam-breaks at opposite ends of the chamber (termed runs) did not differ between genotype (Figure 3.4B, $F_{2,54}= 2.16$, n.s.) and was accordingly greatest during the first session but readily reduced subsequently across

the next sessions (Session 1: $F_{9,488}$= 56.75, p< 0.001; Session 2: $F_{9,487}$= 33.68, p< 0.001; Session 3: $F_{9,506}$= 22.90, p< 0.001, respectively). An analysis of locomotor activity performance was carried out between the WT-control lines, and the results summarised in Appendix 1.3. No difference was found between the WT-P0 mice and WT-Null control mice on either total beam-breaks or runs, across sessions.

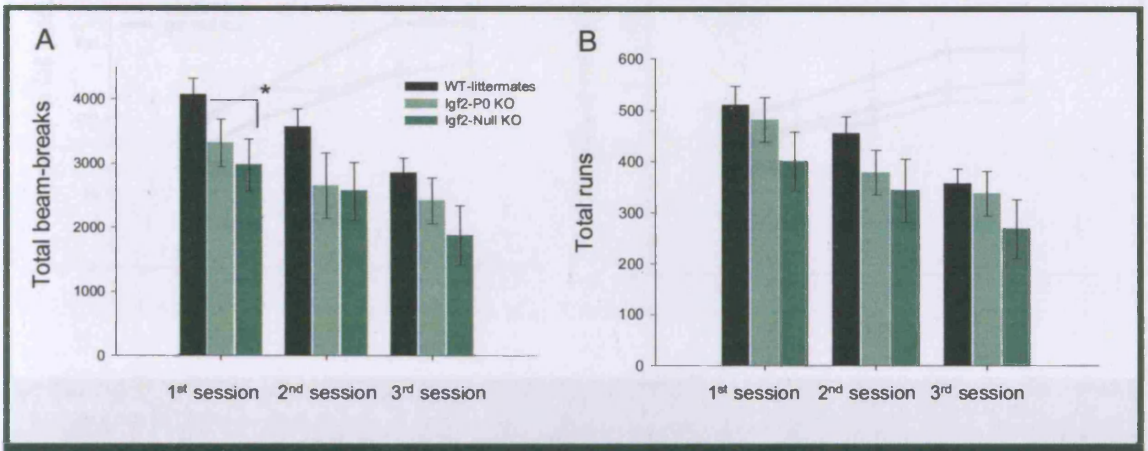


Figure 3.4: *Performance on LMA across experimental sessions.* ***(A)*** *The number of beam-breaks made across the three daily sessions, between mice of different genotypes.* ***(B)*** *The number of two consecutive beam-break made at opposite ends (runs), across the three daily session, between mice of different genotypes. Data are presented as mean values along with SEM.*

**Significant at p<0.05.*

3.3.2.2 Balance and motor control

Mice were given five trials on the rotarod (5-50rpm in 4mins), where subjects of each genotype groups displayed a relatively high degree of motor competence. All subjects showed improved performance on the rotarod test across the five sessions as reflected by increased latency to fall from the rotating bar (Figure 3.5A, within-subject test; $F_{4,217}$= 28.40, p< 0.001) and heightened rotation-speed at the time of fall (Figure 3.5B, within-subject test; $F_{3,214}$= 25.33, p< 0.001). No significant difference in performance was found between the *Igf2*-P0 KO mice and WT-control littermates (*post hoc* test (see ANOVA result below); $F_{1,54}$= 1.07, n.s. and $F_{1,54}$= 1.13, n.s), for latency and rotational speed, respectively). However, mice of the *Igf2*-Null KO line exhibited a greater latency on average to fall from the rod across all five sessions (between-subject test; $F_{2,62}$= 3.60, p< 0.05), although a *post hoc* comparison only found this to be significant relative to the *Igf2*-P0 KO mice ($F_{1,27}$= 7.14, p< 0.05). Accordingly, the *Igf2*-Null KO mice were also shown to fall from the rod at greater

rotation velocities on average (between-subject effect; $F_{2,62}= 3.81$, $p< 0.05$, *post hoc* test; $F_{1,27}= 4.79$, $p< 0.05$), but again this was only significant when compared to KO mice of the *Igf2*-P0 line.

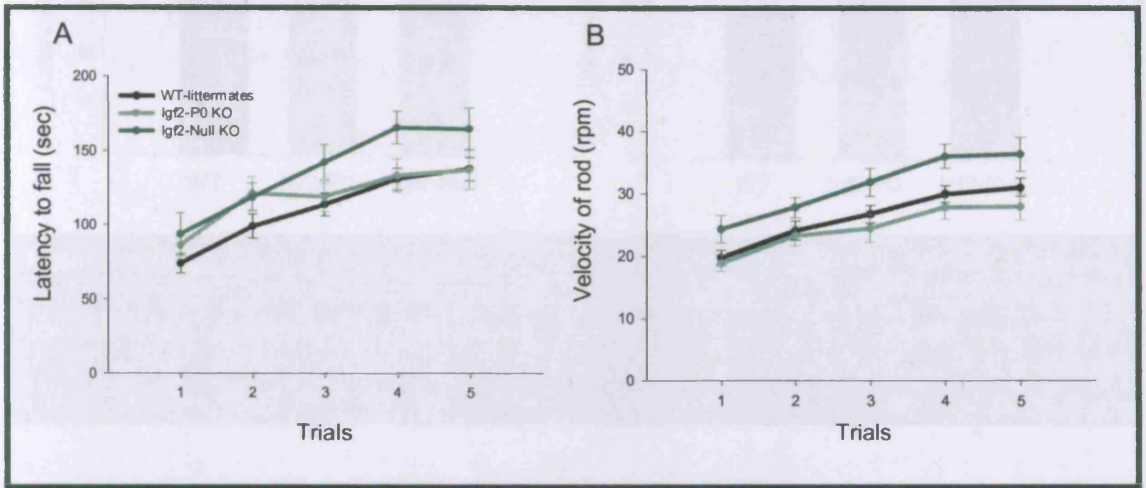


Figure 3.5: *Performance on the rotarod across five trials with over increasing rotation velocity.* ***(A)*** *The latency of subjects from different groups of genotype, to fall of the rod over the five consecutive trials.* ***(B)*** *The velocity level of rod rotation at the time of fall over the five consecutive trials, across mice of different genotypes. Data are presented as mean values along with SEM.*

Conversely, there were no differences revealed between the different groups of mice when subjects were assessed exclusively with one-way ANOVA, either at a rotation velocity of 35rpm (Figure 3.6A, $F_{2,62}= 1.47$, n.s.) or at 45rpm (Figure 3.6B, $F_{2,62}= 2.30$, n.s.). Furthermore, a repeated measures analysis of rotarod performance between the separate WT-control lines showed that WT-controls of the *Igf2*-P0 line stayed on the rod for a longer period of time than WT-Null control mice, before falling (see Appendix 1.4 for summary of results). Similarly, the WT-P0 controls fell from the rod at a significantly greater rotation velocity, than the control mice of the *Igf2*-Null line. However, this was perhaps not surprising considering the increased body weight and size of the WT-Null control mice.

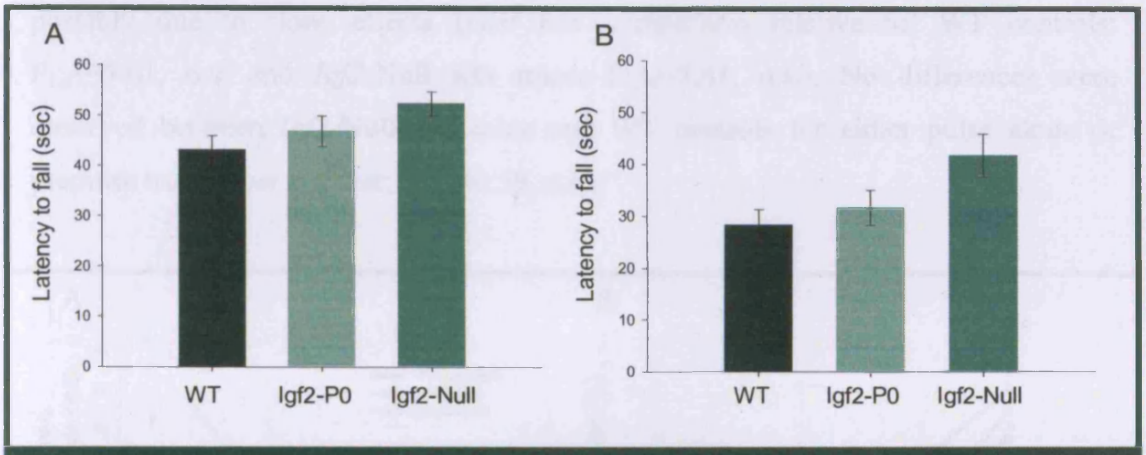


Figure 3.6: *Performance on the rotarod across at fixed rotation velocity.* ***(A)*** *The latency of subjects from different groups of genotype, to fall of the rod at a rotation velocity level of 35rpm.* ***(B)*** *The latency of subjects from different groups of genotype, to fall of the rod at a rotation velocity level of 45rpm. Data are presented as mean values along with SEM.*

3.3.2.3 Acoustic startle and prepulse inhibition

The effects of auditory acoustic stimuli inducing a startle response and a measure of sensory-motor gating were tested in *Igf2*-KO and WT-control mice. Sensory-motor gating was examined by presentation of a range of prepulse stimuli prior to the startle stimuli, inducing a phenomenon known as prepulse inhibition (PPI), whereby the amount of startle reflex behaviour is attenuated (Li et al., 2009). PPI is characterized as a form of low level perceptual gating. The amount of movement induced by the startle stimuli (startle reflex) was determined by the piezoelectric response to the flexion in the base of the chamber in which the mice were placed. Due to the differing body weight of the different genotype models under test, most notably that of the *Igf2*-Null KO mice, the reported values for startle reflex (in arbitrary startle units) were normalised to $(\text{body weight})^{0.75}$ (see section 3.2.3.3).

Mice of all genotypes demonstrated a startle response to the 120dB stimulus (Figure 3.7A). However, the magnitude of the startle response to the acoustic stimulus (pulse alone) was found to be significantly elevated for *Igf2*-P0 KO mice (one-way ANOVA; $F_{2,81}= 8.67$, $p< 0.001$), relative to *Igf2*-Null KO mice (*post hoc* test: $F_{1,34}= 7.02$, n.s.) and WT-control littermates (*post hoc* test: $F_{1,67}= 17.08$, $p< 0.001$). This effect was still present on trials where the startle stimulus was preceded by prepulse stimuli at 2, 4 and 8 dB above background (70dB) (interaction: $F_{1,81}= 33.04$, $p<0.001$), but failed to reach significance at a prepulse level of 16dB above background,

possibly due to floor effects (*post hoc* comparison relative to; WT controls: $F_{1,67}$=5.61, n.s. and *Igf2*-Null KO mice: $F_{1,34}$=5.61, n.s.). No differences were observed between *Igf2*-Null KO mice and WT controls for either pulse alone or prepulse trials (*post hoc* test; $F_{1,61}$=0.39, n.s.).

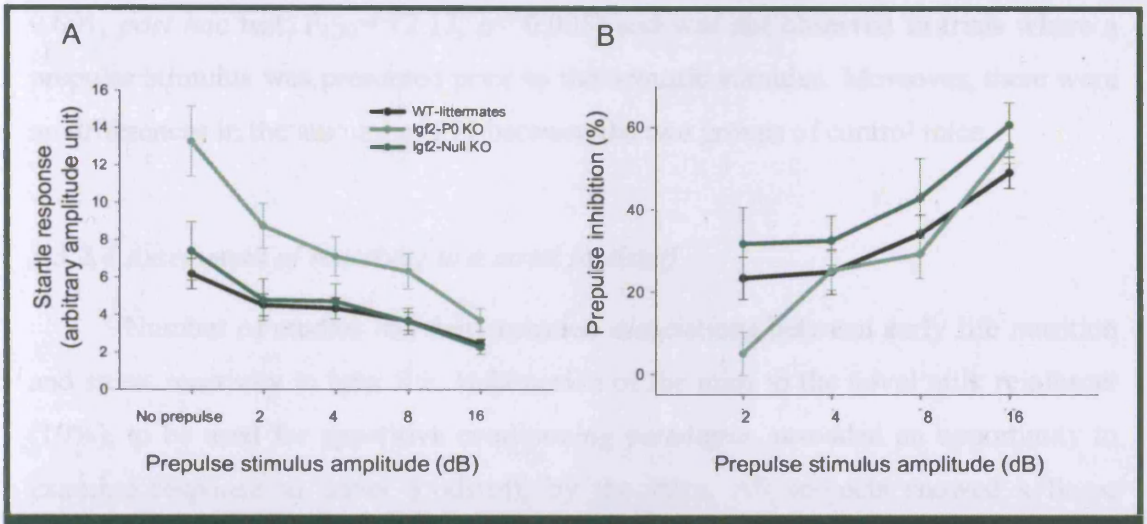


Figure 3.7: *Startle reactivity and PPI.* ***(A)*** *The amplitude of startle response emitted by mice of different genotypes (after adjustment for body weight), across varying sound levels of the prepulse stimulus* ***(B)*** *The degree of prepulse inhibition (in terms of percentage) across varying sound levels of the prepulse stimulus, between mice of different genotypes. Data are presented as mean values along with SEM.*

All mice demonstrated a pattern of reduced startle responses as the amplitude of the prepulse increased (Figure 3.7A, within-subject test; $F_{2,140}$= 52.57, p< 0.001), a trajectory that did not differ between the different genotypes (between-subject test; $F_{1,81}$= 33.04, p< 0.001). However, as noted, the heightened startle response of the *Igf2*-P0 KO mice persisted through this manipulation (see above). Calculation of PPI, the degree with which the startle response is reduced by the prepulse, showed a general increase with increasing amplitudes of the prepulse stimuli (Figure 3.7B, within-subject test; $F_{3,243}$= 33. 34, p< 0.001) for all groups of mice (between-subject test; $F_{2,81}$= 1.33, n.s.). However, closer examination of the data showed that *Igf2*-P0 KO mice did not exhibit PPI when the prepulse was 2dB above background (Student's paired-sample) T-test; $t(20)$= 0.94, n.s.) in contrast to the *Igf2*-Null KO and WT-control mice which both showed significant PPI ($t(14)$= 3.54, p= 0.005) and $t(47)$= 4.57, p= 0.001, respectively), suggesting a degree of altered sensory-motor gating in *Igf2*-P0 KO mice.

There was a significant difference noted between the separate WT-littermate control groups in acoustic startle response following presentation of pulse-alone stimuli (as summarized in Appendix 1.5). This was in terms of greater magnitude of response for WT-control mice of the *Igf2*-P0 mutant line, however, this response was still significantly different from *Igf2*-P0 KO mice (one-way ANOVA; $F_{3,80}= 7.10$, $p< 0.001$, *post hoc* test: $F_{1,30}= 12.13$, $p< 0.005$) and was not observed in trials where a prepulse stimulus was presented prior to the acoustic stimulus. Moreover, there were no differences in the amount of PPI between the two groups of control mice.

3.3.2.4 Assessment of reactivity to a novel foodstuff

Number of studies has demonstrated associations between early life nutrition and stress reactivity in later life. Habituation of the mice to the novel milk reinforcer (10%), to be used for appetitive conditioning paradigms, provided an opportunity to examine response to novel foodstuff, by the mice. All subjects showed a linear increase in total liquid consumption across six habituation sessions (Figure 3.8A, within-subject test; $F_{4,533}= 114.24$, $p< 0.001$), following a Kleiber's 0.75 mass exponent method for adjustment of body weight. However, no difference was found in consumption between genotype groups (between-subject test; $F_{2,120}= 2.15$, n.s.). Importantly, a one-sample T-test showed that all subjects displayed equivalent consumption of water across the two containers during the first two sessions (*Igf2*-P0 KO mice; Session 1: $t(35)= -0.55$, n.s., Session 2: Day1: $t(35)= -0.72$, n.s.; *Igf2*-Null KO mice; Session 1: $t(12)= -1.26$, n.s., Session 2: $t(12)= -0.95$, n.s.; WT controls; Session 1: $t(66)= -0.05$, n.s., Session 2: $t(66)= -0.28$, n.s.), indicating that the subjects did not make any discrimination in their water consumption between the locations of the containers.

Furthermore, although the WT controls showed a significant preference for condensed milk from the first session of preference testing between condensed milk and water (i.e. relative to 50%, Session 3, $t(66)= 3.21$, $p< 0.002$), the mice of the *Igf2* KO lines did not display milk preference until the second session of preference testing (Session 4; *Igf2*-P0 KO: $t(35)= 22.24$, $p< 0.001$, *Igf2*-Null KO: $t(19)= 4.54$, $p< 0.001$). Also of interest, a repeated measures analysis reported a significantly lower preference for the novel milk solution among *Igf2*-P0 KO mice during Session 3 (Figure 3.8B, one-way ANOVA; $F_{2,120}= 4.67$, $p< 0.02$), in comparison to milk

preference of the WT-control littermates (*post hoc* test: $F_{1,101} = 4.67$, $p < 0.011$), albeit this difference did not persist in Session 4 (one-way ANOVA: $F_{2,120} = 1.47$, n.s.) or in subsequent sessions (Session 5: $F_{2,120} = 0.21$, n.s.; Session 6: $F_{2,120} = 1.27$, n.s.).

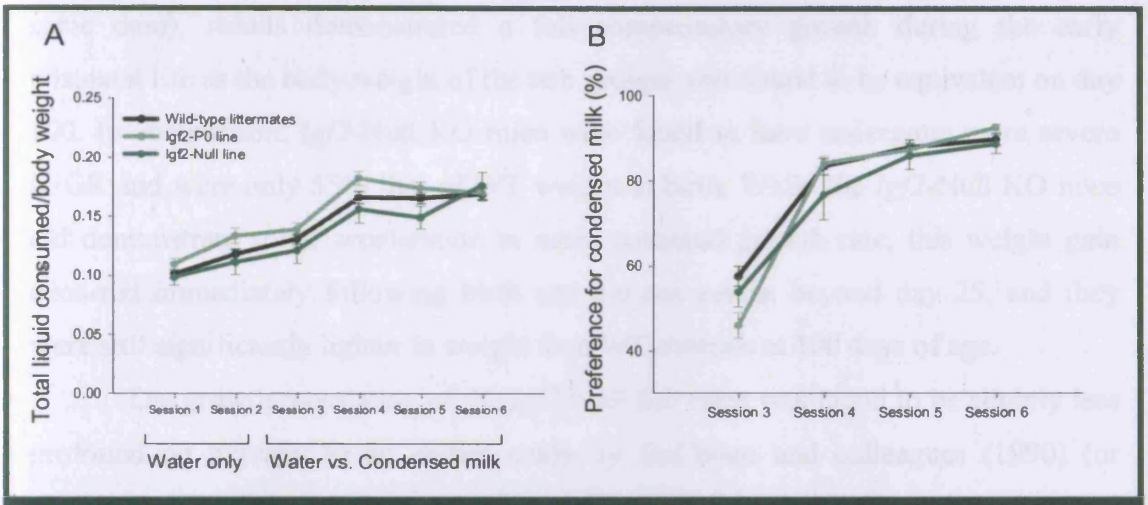


Figure 3.8: *Food neophobia and habituation.* ***(A)*** *The total volume of liquid consumed, between mice of different genotype groups, across sessions (after adjustment for bodyweight).* ***(B)*** *The preference for the novel foodstuff (10% condensed milk) between mice of different genotypes, across sessions. Data are presented as mean values along with SEM.*

In contrast, the *Igf2*-Null KO mice did not demonstrate any difference in the condensed milk preference from WT controls (*post hoc* test; $F_{1,85} = 0.63$, n.s.) or *Igf2*-P0 KO mice (*post hoc* test $F_{1,54} = 2.37$, n.s.) during the first preference session, or subsequent sessions ($F_{2,120} = 0.45$, n.s.). Finally, a separate analysis of condensed milk preference and the total volume of liquid consumed between the littermates of the two control-lines did not shown any difference on either parameter, as summarized in Appendix 1.6.

3.4 Discussion

This initial series of experiments investigated the early growth characteristics, development and sensory/motor behaviour of *Igf2* KO mice. In the present study, both deletion of the *Igf2* gene of the sole placental specific transcript (*Igf2*-P0) and complete deletion of all of the foetal and placental transcripts (*Igf2*-Null), resulted in considerable foetal growth deficiency in mice consistent with previous findings (Constância et al., 2002; DeChiara et al., 1990). The *Igf2*-P0 KO mice displayed

moderate IUGR, in terms of reduction of birth weight to 75% that of WT littermates, followed by an early compensatory weight gain between postnatal days 25-75, leading to less than 7% difference in adult body weight by postnatal day 100. However, when *Igf2*-P0 KO mice were compared solely to their own WT littermates (i.e. from the same dam), results demonstrated a full compensatory growth during the early postnatal life as the body weight of the two groups were found to be equivalent on day 100. In comparison, *Igf2*-Null KO mice were found to have undergone more severe IUGR and were only 55% that of WT weight at birth. While the *Igf2*-Null KO mice did demonstrate some acceleration in early postnatal growth rate, this weight gain occurred immediately following birth and did not persist beyond day 25, and they were still significantly lighter in weight than WT controls at 100 days of age.

The growth retardation of the *Igf2*-Null KO mice was found to be slightly less profound on average to an earlier study by DeChiara and colleagues (1990) (or approximately 60% of wild-type birth weight). In addition, no difference was noticed in early postnatal growth rate between the *Igf2*-Null mice and WT littermates in that study, suggesting that the early catch-up growth might be a physiological response to the imbalance between the nutritional supply and demand rather than due to the growth restriction as such. The *Igf2*-Null KO mice were also found to be delayed on all four morphological measurements of somatic development (i.e. unfolding of ear pinna, incisor eruption, bilateral eye opening and fur growth), however this was not the case for mice of the *Igf2*-P0 KO line.

One of the main findings from the behavioural experiments, was that the *Igf2*-P0 KO mice displayed significantly elevated startle responses in the startle and PPI paradigm (i.e. relative to the WT littermates) and greater neophobic response to novel food-stuff, evidence of heightened stress reactivity/anxiety-related behaviour (Rodríges-Fornells, Riba, Gironell, Kulisevsky and Barbanoj, 1999; Zhu et al., 2006, respectively). In addition, the *Igf2*-P0 KO mice also failed to show PPI at prepulse amplitude level of 2dB, possibly as a consequence of a mild impairment in sensory-motor gating. In contrast, the *Igf2*-Null KO mice did not show any differences from WT-littermate performance on either the startle and PPI paradigm, or the food neophobia test. These results are particularly noteworthy, considering the fact that PPI has been demonstrated to be attenuated in several different anxiety-related disorders (Duley et al., 2007), and similar increases in stress responses and anxiety-related behaviour have also been described in a number of animal models, using alternative

means of inducing intrauterine malnutrition (i.e. reduction in maternal food intake or dietary manipulations) (Levay et al., 2008; Erhard et al., 2004; Jaiswal, 1996), epidemiological studies of people born with low birth weight (LBW) or small for gestational age (SGA, see Chapter IV, section 4.1 for further discussion of this evidence) and studies showing the association between IUGR and hyperactivation of the hypothalamic-adrenal-pituitary (HPA) axis in both animals and humans (see Chapter IV, section 4.4). Moreover, as previously mentioned in section 3.1, impaired sensory gating has also been shown to be a common feature in ADHD and schizophrenia, highlighting that further behavioural investigation is warranted, as behavioural tests sensitive to ADHD and schizophrenic related impairments could perhaps enable further dissociation of effects in *Igf2*-P0 KO mice with respect to *Igf2*-Null KO and WT controls (see also section 1.2.1).

It is possible that the reluctance of the *Igf2*-P0 KO mice to consume the condensed milk might have stemmed from developmental alterations in either motivational or sensory properties. What undermines the feasibility of such explanations however is the fact that the *Igf2*-P0 KO mice only showed this reduced milk preference during the first day of being provided with milk-water option, whereas by the second day the preference for the condensed milk was indistinguishable from that of WT littermates and *Igf2*-Null KO mice. Nevertheless, in order to provide further support for an anxiogenic explanation for this difference and for the other behavioural phenotypes found, further investigation of anxiety-related behaviour among the *Igf2*-P0 KO mice would be of relevance.

There were no differences found between *Igf2*-P0 KO mice and WT controls in measures of LMA or motor coordination, as tested on the rotarod. When tested on the LMA chambers, the *Igf2*-Null KO mice showed a general decrease in ambulation (in terms of fewer beam-breaks), but this reduction was only found to be significant during the first session of testing. On the other hand, the *Igf2*-Null mice also exhibited an improved overall performance on the rotarod apparatus, indicated by greater latency of the *Igf2*-Null KO mice to fall from the rotating rod, as well as heightened rotational speed at the time of fall. While the improved rotarod performance could easily be attributed to the relatively smaller body size of the *Igf2*-Null KO mice (i.e. due to smaller body/rod size ratio, similar pattern of effects shown by chromosome X imprinting centre (IC) deletion mice, also of small size relative to controls, Relkovic et al., 2010), the reason behind the decreased ambulation on the LMA chambers is

less clear. This reduction in LMA could reflect impaired motor ability by *Igf2*-Null KO mice, however motivational or emotional aspects must also be taken into consideration. The role of stress or anxiety-related properties underlying this reduction in LMA could possibly be excluded with further examination on test-batteries of anxiety-related behaviour.

A key aspect in analysing data from these studies, concerns the equivalence of the two groups of WT-control mice. Although of equivalent background strain, age, and husbandry, the differences observed could make grouping of the data problematic. Notable differences were observed in both postnatal growth trend and somatic development, across the segregated WT-littermate control groups, where littermate controls from the *Igf2*-Null mutant line reached a slightly greater body weight at day 100 and also displayed a delay on the somatic indices of ear pinna unfolding and incisor eruption. Since the birth weight of the two WT-control groups was found to be the same, it is plausible that these marked differences in early postnatal development reflect variations in the early postnatal environment (although disparity in intrauterine environment should not be excluded). This may have arisen from differential access to early postnatal nutrition, as the WT-Null controls are likely have gained greater access to the maternal milk due to weaker competition from their much smaller KO littermates. Alternatively, other early life environmental factors could have attributed to the differential postnatal development of the WT-littermate controls, such as difference in maternal or KO littermate behaviour towards the two WT-littermate groups. Perhaps more importantly, the WT littermates of the *Igf2*-P0 line produced greater startle response during the acoustic startle and PPI paradigm, when the prepulse stimulus was absent, although direct comparison with *Igf2*-P0 KO mice did not affect the significance in this case. The WT-P0 controls also demonstrated a superior overall performance on the rotarod apparatus in comparison with WT-Null littermates, however this difference is likely to be attributed to greater body weight (and size) of the WT-Null mice (in terms of potential increase in body-rod size ratio).

As mentioned earlier in Chapter II (section 2.2.7), the aforementioned differences between the segregated WT-littermate control groups raises a serious concern regarding the methodological validity of pooling the two WT-control littermate groups. This is because any variations between the pooled WT-control groups (particularly in terms of behaviour) could significantly distort the results from a between-genotype comparison when using a single collective WT-control group as

baseline. Firstly, an irregularly large variation between the two WT-control groups will undoubtedly increase the within-group variance of the collective WT-control group, and thus reduce the sensitivity of a given statistical test to identify difference in variation between groups. Secondly, due to the fact that littermates from one of the WT-control groups might differ in one or more ways from what constitutes 'normal' behaviour for mice of this particular background strain generated in the absence of an genetically modified littermates (e.g. due to unfavourable prenatal or postnatal environmental influences), it is possible that the behavioural data from one (or both) of these groups of mice could skew the comparative WT-control baseline from what presumably is 'normal' WT behaviour (i.e. if the skewness goes in opposite direction from the variance of the *Igf2* KO mouse models). Hence, any apparent difference in behaviour observed among KO mice of either *Igf2* mouse models could in fact be false, as the baseline does not accurately represent normal WT behaviour. However, although the WT littermates from *Igf2*-P0 control group are more likely to correspond to normal WT mice than WT-Null control mice (by observation, in relation to body weight and somatic development), this is difficult to conclude with certainty. In spite of the fact that the two different groups of WT-littermates were found to be significantly different for some measures, it is also plausible that this between-group difference might lie within the range of what could constitute normal variation in both physical and behavioural properties of regular WT mice. Furthermore, carrying out a comparative analysis of behaviour between mice of the *Igf2* KO mouse models and WT littermates using two segregated WT-control groups instead of a single collective WT-control group would make data interpretation both lengthy and perhaps at times confusing. As such, the approach of using two segregated WT-control groups could produce behavioural differences which are irrelevant to the purpose of the current thesis. Therefore, in order to confront the compromised methodological validity of employing a pooled WT-control group, it is crucial to ensure that for any difference observed between mice of either one or both of the *Igf2* KO models, and the collective WT-control group, there is no difference is found between the segregated WT-control groups on that parameter. Moreover, it would also be relevant to carry out individual comparison across mice of a give *Igf2* KO group and WT littermates from separate control groups (particularly the WT group of the same breeding line), for any difference obtained between the *Igf2* KO mice and the collective WT controls, in effort to evaluate the strength and the nature of this difference. Conceivably, this

would enable a reliable and valid inference of any behavioural difference as a result of placental deficiency and IUGR, rather than irregularity of WT-littermate behaviour. This will be further addressed in the general discussion in Chapter VIII.

The findings from the present study provide for the first time evidence for compromised brain development and behaviour among mice of the *Igf2*-P0 KO model, as indicated by the marked differences in stress and anxiety-related phenotypes. Although, there was a notable difference found among the *Igf2*-Null KO mice on behavioural tests involved with competence of locomotor activity, balance and motor coordination, it remains unclear whether this is due to differences in early brain development or in other physical/morphological aspects related to their small size. Furthermore, it is important to note that the differential behavioural profile described between the *Igf2*-P0 and *Igf2*-Null KO mice, may reflect dissociation between consequences of nutrient deficiency (due to compromised placental function) and growth restriction, in intrauterine life (see Chapter I, section 1.6). As such, it is conceivable that the heightened stress/anxiety-related behaviour might be a result of foetal malnutrition, rather than IUGR. However, this topic will be subjected to further discussion in the General Discussion (Chapter VIII, section 8.3). The studies described in the present chapter are the first to describe the adaptation of a genetic model to mimic and investigate the long term deleterious consequences of adverse intrauterine environment, on brain development and behaviour. Still, further research into the cognitive functions and behaviour of the *Igf2* KO mouse models, is essential, in order to gain further converging (and thus stronger) evidence for heightened stress and anxiety among these mice, as well as to establish a broader characterization of their cognitive and behavioural domains.

3.4.1 Summary of key results from Chapter III

- KO mice of both *Igf2*-P0 and *Igf2*-Null lines showed considerable IUGR as indicated by the lowered birth weight, although this growth deficiency was found to be more severe in *Igf2*-Null KO mice.
- *Igf2*-P0 KO mice showed a considerable compensatory weight gain during early postnatal life (day 25-50), and displayed normal development across all somatic indices.
- *Igf2*-Null KO mice showed a transient growth spurt in the first few days following birth (day 0-25) and were delayed on all measurements of somatic development and remained growth impaired throughout life.
- *Igf2*-Null KO mice showed an overall reduction in ambulation in the LMA chambers relative to *Igf2*-P0 KO mice and WT controls, although a significant difference was only obtained on the first session. In contrast, no difference was found between *Igf2*-P0 KO mice and WT controls.
- *Igf2*-Null KO mice displayed an overall improvement in motor co-ordination and balance on the rotarod apparatus relative to the *Igf2*-P0 KO mice, as indicated by greater latency to fall from the rod and a greater rotation velocity at the time of fall.
- *Igf2*-P0 KO mice showed significantly greater startle amplitude in response to acoustic stimuli than *Igf2*-Null KO and WT controls. This effect still persisted during trials when the startle stimulus was preceded by prepulse stimuli at 2, 4 and 8 dB above background, but not at 16dB.
- *Igf2*-P0 KO mice showed a mild impairment in PPI during trials when the PPI was 2dB above background.
- *Igf2*-P0 KO mice showed a significantly reduced preference for a novel foodstuff during the first day of testing relative to *Igf2*-Null KO and WT controls.

Chapter IV

Investigating Stress/Anxiety-Related Behaviour in Mice of the Igf2-P0 and Igf2-Null Knockout Lines: Elevated Plus-Maze, Open Field and Free-Exploratory Paradigm

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4.1 Introduction

Pathological anxiety is the most prevalent psychiatric disorder in the general population, with lifetime prevalence approximating 29% (Kessler et al., 2005). In recent years, there has been emerging evidence indicating that early life factors might play a role in the risk of developing stress and anxiety-related disorders in later life. Studies have shown different relationships between birth weight and measures of stress and anxiety, such as an inverse correlation between birth weight and stress susceptibility (Nilsson et al., 2001) or an inverted 'U' function, whereby individuals at both the lower and higher ends of the birth weight distribution were at greater risk of having anxiety-related symptoms in adulthood (Alati et al., 2009). Moreover, these patterns have been replicated in studies of low birth weight (LBW) children that were born full-term (Gustafsson, Josefsson, Selling and Sydsjö, 2009; Wiles, Peters, Leon and Lewis, 2005; Cheung, Khoo, Karlberg and Machin, 2002). The associations of foetal growth with later anxiety were shown to persist, when adjusted for socioeconomic status in order to control for effects of continuing postnatal adversity (Cheung et al., 2002; Wiles et al, 2005; Nilsson et al., 2001). Physiological differences have also been found, such as higher resting heart rates and elevated urinary catecholamine levels among children that have been growth retarded from early childhood, relative to nonstunted counterparts (Fernald and Grantham-McGregor, 2002; Fernald and Grantham-McGregor, 1998). However, a key issue is the association between measures of foetal growth or birth weight as accurate predictors of an adverse foetal environment, as discussed in Chapter I (section 1.5.1). Thus, results from the epidemiological studies may only represent summary indices of successful foetal and infant development, which itself is not causally related to the long-term effect on behaviour and mental health (Schlotz and Philips, 2009; Gillman, 2002). Therefore, it still remains unclear whether the associations reported in these

studies reflect foetal under-nutrition, or other factors that contribute to impaired foetal growth and later risk of anxiety-related disorders.

The notion of early nutritional origins affecting adult stress/anxiety-related behaviour has also gained some support from work utilising animal models, although the overall findings from this line of research are ambiguous, and in some regards contradictory. Adult rats exposed to under-nutrition during gestation or lactation showed heightened anxiety when assessed using two common rodent paradigms purported to assay anxiety, the elevated plus maze and the open field test (see below for test details) (Jaiswal et al., 1996; Levey et al., 2008). Pain thresholds and emotional reactivity has also been found to be elevated in rats (Smart et al., 1973; Levitsky and Barnes, 1970) and pigs (Barnes, Moore and Pind, 1970), which had received inadequate nutrition during early postnatal life. In further support of the behavioural results in rodents, adult sheep showed enhanced emotional reactivity when exposed to restraint or novel stimuli, when their mothers received a protein restricted diet during early gestation (Erhard et al., 2004).

In contrast, several other studies have been unsuccessful in demonstrating a link between early nutritional deficits and stress- or anxiety-related behaviour in adulthood. In rats, one such study demonstrated that the imposition of a protein-calorie restricted diet during gestation did not produce any difference on anxiety/stress related parameters when tested on the open field (Villescas et al., 1981), and some studies have even noted a reduction in anxiety among rats that were malnourished *in utero* (Almeida et al., 1996a; Almeida et al., 1996b). Additionally, a number of studies have also reported anxiolytic effects of early postnatal under-nutrition (Frangolin-Silva, Hernandes, Fukuda, Valadares and Almeida, 2006; Almeida, Garcia and De Oliveira, 1993; Almeida, De Oliveira and Graeff, 1991; Brioni and Orsingher, 1988), but the results of Erhard et al. (2004) in juvenile lambs could not be replicated (Simitzis et al., 2008).

There are a number of possible explanations that could either individually, or in combination, account for some of the spurious and contradicting results that have arisen from the animal work. Firstly, the different procedures employed to induce malnutrition, and secondly, the severity of the nutritional manipulation, may yield very different outcomes in adult assessments of stress and anxiety. Third, animal studies have involved the use of nutritional manipulation during different stages of development, both during gestation and in the early neonatal time, which may lead to

profound differences in neurobiological and behavioural phenotypes in later life. Finally, and perhaps of fundamental importance, some of the aforementioned animal studies may have been prone to methodological limitations, for reasons such as; lack of standardized tools for investigating stress/anxiety-related phenotypes, or insufficient efforts to control for possible confounds. For instance, it is difficult to conclude whether the behavioural differences observed are in fact a result of foetal under-nutrition, or rather due to differences in maternal care-taking as result of maternal food restriction, as has been previously discussed (see Chapter I, section 1.5.2, for more detailed discussion). These variations in experimental methodology and control make a clear interpretation of the findings from animal models particularly difficult, and suggest that the current experimental evidence for a causal relationship between intrauterine nutrition and later risk of anxiety disorders is relatively weak.

In Chapter III, mice of the intrauterine growth restricted (IUGR) *Igf2*-P0 KO line displayed a greater startle response to acoustic stimuli, as well as a reluctance to consume a novel food. As discussed, these finding might suggest heightened anxiety in adulthood, as a result of placental insufficiency-induced IUGR. Nevertheless, further evidence is warranted in order to provide better support for this notion. Therefore, to extend the findings of Chapter III, the main aim of this chapter was to explicitly investigate the impact of placental deficiency and IUGR on anxiety-related behaviour among mice of the *Igf2*-KO lines. Thus, a number of unconditioned conflict tests of anxiety-related behaviour (termed approach-avoidance conflict tests) were carried out on the *Igf2*-KO mice and WT-littermate controls: the elevated plus maze (EPM), open field (OF) and the free-exploratory paradigm (FEP). These paradigms of anxiety-related behaviour are based on the natural conflict observed in rodents between the drive to explore a new environment and the tendency to avoid a potentially dangerous region (Ramos, 2008), and have been shown to be reasonably sensitive to classical, anxiety-reducing, benzodiazepine drugs (see Lister, 1990 for review; Belzung and Berton, 1997). The EPM is the most widely used test of anxiety-related behaviour in animal models (Carobrez and Bertoglio, 2005; File, Lippa, Beer, Lippa, 2004) and involves assaying the aversion of rodents to open space and height. The maze has four arms where two opposing arms are enclosed by high walls and the other two have no sides. An index of anxiety is determined by measuring the accumulated time and frequency of entries by subjects onto the open arms of the maze

relative to the enclosed arms. The OF, another commonly used test of anxiety-related behaviour, is a large and brightly lit open arena from which escape is obstructed by high walls. Rodents with high levels of anxiety usually spend more time close to the peripheral walls of the arena (thigmotaxis) whereas those of low anxiety levels will freely explore the arena, frequently entering the central region. The FEP assesses anxiety in terms of the response of an animal to a novel environment (neophobia). Subjects are habituated to one side of a two compartment place preference apparatus before being allowed to explore the other, novel, side. Rodents with high anxiety levels usually spend lesser time in the novel compartment relative to non-anxious rodents (Teixeira-Silva et al., 2009).

Due to the heterogeneity of anxiety (Unick, Snowden and Hastings, 2009), it is essential that multiple tests of anxiety-related behaviour are used in order to broadly assess the different psycho-physiological states that might be reflected by these tests. In addition to the bidirectional sensitivity of anxiolytic/anxiogenic drug manipulations on behaviour, these ethologically based conflict tests of anxiety provide the advantages of not requiring lengthy training procedures, the use of food/water deprivation or the conditioning of aversive stimuli (Rodgers, Cao, Dalvi and Holmes, 1997). Furthermore, since these tests do not involve a conditioning procedure, there is no (or at least less) reliance on memory or attention and confounding effects of activity can be taken into account.

The collective results from these tests of anxiety-related behaviour could provide further evidence for the putative anxiogenic effects of foetal malnutrition and growth deficiency, on mice of the *Igf2*-P0 knockout (KO) line. Although the reviewed literature does not provide a clear hypothesis as to the direction of altered anxiety that might be expected to be demonstrated by *Igf2*-P0 KO mice relative to *Igf2-Null* and wild-type (WT) mice, the evidence to date (including that from Chapter III) does suggest that *Igf2*-P0 KO mice may show some specific anxiety-related effects.

4.2 Materials and Methods

Full methodological descriptions can be found in the relevant sub-sections of the General Methods Chapter (Chapter II).

4.2.1 Subjects and animal husbandry

Two cohorts of age-matched (8 to 12 months old at the onset of testing) adult male mice (generated in the Behavioural Neuroscience Laboratory at Cardiff University) were used in this series of experiments (see Table 3.1). The first cohort of mice was subjected to testing on the EPM and OF. The EPM preceded testing in the OF and a number of mice were removed from the study for a number of different reasons including, tissue collection and illness. A second group of mice was tested on the FEP. Mice were obtained from a crossbreeding of background strain of C57BL/6*129 (carrying either the mutant *Igf2*-P0 allele or the *Igf2*-Null allele) with CD1 female (as previously described in Chapter II). The mice were housed in littermates groups of two to five animals per cage, under temperature- and humidity-controlled conditions, with a 12-hour light: 12-hour dark cycle (lights on at 07:30). All subjects had *ad libitum* access to standard laboratory chow and water and were weighed on regular basis. The body weight of each mouse was monitored regularly for indication of health status (see Chapter II, section 2.4.2). All experimental procedures were conducted under licenses issued by the Home Office (U.K.) in compliance with the Animals (Scientific Procedures) Act 1986 and local ethics committee.

Table 4.1: *The sample size and genotype of each cohort-subset of mice assayed on ethologically-based conflict tests of anxiety: EPM, OF and FEP.*

Behavioural Apparatus	Genotype and sample size (*n*)		
	Igf2-P0	*Igf2*-Null	WT (WT-P0/WT-Null)
Elevated plus maze	21	14	47 (23/24)
Open field arena	17	11	42 (18/24)
Free exploratory paradigm	18	9	40 (22/18)

4.2.2 Behavioural methods

4.2.2.1 General methods

Each of the test methods used to assess anxiety involved individual testing of mice; hence the apparatus was cleaned thoroughly with a 2% acetic acid solution between subjects, to remove odours possibly left by the previous subject. All testing took place between the hours of 10:00 and 17:00, with equal distribution of testing for subjects of different genotypes throughout the day, and prior to testing mice were habituated to the test room for at least 20 minutes. The test room was illuminated by a dim light and care was taken to minimise physical disturbance or excessive noise. Data for the EPM, OF and FEP were collected using EthoVision Observer software (Version 3.0.15, Noldus Information Technology, Netherlands) with specific virtual arena and zone configurations for each piece of apparatus (see below). The EthoVision tracking system performs calculations over a series of frames (12 frames/sec) to derive a set of quantitative descriptors about the movement of subjects, and variables such as duration and entries into the arms were determined by the EthoVison programme in terms of location of the greater body-proportion of subjects. Tracking of the subject was calibrated for each piece of apparatus prior to testing using non-experimental mice of the same body size and fur colour as the experimental subjects. Data were analysed as total values for each session and in 1-minute intervals to assess performance within a session. Each session was also recorded using DVD HD recorders (Sony Corp, U.K.) for further analysis if required.

4.2.2.2 The elevated plus maze paradigm

In order to assay the innate aversion of both lines of *Igf2*-KO mice to open space and height, relative to WT-littermate controls, the subjects were placed at the centre of the EPM (as previously described in Chapter II, section 2.3.4) facing one of the exposed open arms and allowed to explore the maze over a single session of 10 minutes. The maze was illuminated by a single 60 Watt lamp, located 75cm above the central region of the maze. To optimize data analysis, the different parts of the EPM was divided into 5 virtual zones representing the 2 open arms, 2 closed arms and the middle section from which all arms originated, using the EthoVision Observer software. 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. Therefore the

following data were collected for each cumulative zone: accumulated time, latency of first entry, frequency of entries, duration of movement, average velocity and distance moved. In addition, the following parameters were manually scored: number of rears (defined as standing on hind feet only), 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). Data were expressed for the total session and in 1-minute time intervals (or blocks). 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), the number of entries onto the open arms and the latency of first entry onto the open arms. Other potential indicators of anxiety states included; the number of head dips on the unprotected open arms, the number of rears and SAPs. The parameters used as measurements of locomotor activity were; the total duration of movement, the average velocity of locomotion, as well as the distance moved.

4.2.2.3 The open field paradigm

Mice of the *Igf2*-KO lines were tested on the OF, a white square shaped Perspex arena enclosed by high walls (see chapter II, section 2.3.5 for further details of the apparatus), along with WT-control littermates, in an effort to investigate possible differences across the subjects in their aversion to unprotected open areas. The maze was illuminated by a single 100 Watt lamp, located 75cm directly above the central region. At the start of the 20-minute test, the mice were placed, facing the wall, at the edge of the arena at a point equidistant along the wall. The key measure of anxiety-related behaviour in the OF is the proportion of time in the central part of the arena relative to the more peripheral areas, thus the EthoVision tracking software programme was applied to subdivide the arena into two virtual concentric squares with dimensions of 200*200mm ('inner-square')/ 400*400mm ('middle-square') and remainder peripheral area ('outer region'). The following tracking parameters were calculated for each of the designated regions of the arena and the arena as a whole: accumulated time, latency of first entry, frequency of entries, duration of movement, average velocity and distance moved. Data were expressed for the total session and in 2-minute time intervals. The main parameters used as indices of anxiety-related

behaviour were the proportion of time spent exploring the central regions (relative to the total time spent in the arena), the frequency of entries into the inner-square and the latency to first entry into the inner-square. The parameters used as measurements of locomotor activity were; total duration of movement, the average velocity of locomotion, the distance moved and the number crossings of quadrant lines.

4.2.2.4 The free-exploratory paradigm

The *Igf2*-KO mice and WT-controls were tested on the FEP in order to provide an alternative measurement of potential differences in anxiety-related behaviour among the mice. In the FEP, the level of aversion among mice to novel environment is examined, by allowing the subjects to freely explore an environment that contains both familiar and novel parts. The chamber was illuminated by a single 100 Watt lamp, located 75cm above the central region. Subjects were placed, in the centre facing away from the communicating door, of one of two possible compartments of the FEP apparatus (as previously described in Chapter II); a white coloured compartment with smooth floor surface or a black coloured compartment with a coarse-grained floor surface (i.e. coarse sandpaper); the colours and textures of the two compartments was varied to enhance discriminability of the two environments. The chamber used for each subject was counter-balanced between genotypes. Subjects were allowed to move freely within the selected compartment for a 45-minute habituation session and the communicating door to the other compartment was blocked, preventing access. Following the habituation session, the mouse was removed from the apparatus and placed in a holding cage whilst both of the chambers were thoroughly cleaned with 2% acetic acid solution. The communicating door was opened and the mouse was replaced into the centre of the habituated chamber, facing away from the doorway and allowed to freely explore the entire apparatus, i.e. both the familiar and the novel compartment, for a 10 minute (free exploration) test session. In addition to the cleaning with 2% acetic acid solution between subjects as mentioned previously, the sandpaper on the floor of the black compartment was replaced between each subject. EthoVision tracking was performed during both the habituation and exploration phases of the experiment. The FEP chamber was subdivided into four virtual equal regions, designated as novel near and far, familiar near and far, where distance was determined by proximity to the communicating door.

Thus, each region had a span of 150 mm. This subdivision has the advantage of increasing the sensitivity of the obtained measurement, as more anxiogenic subjects may be more reluctant to enter the more distal part of the novel chamber, remaining close to the compartment entrance (i.e. in the proximal part). Thus, the following parameters for each region were calculated: accumulated time, latency of first entry, frequency of entries, duration of movement, average velocity and distance moved. Data were expressed for the total session and in 1-minute time intervals. The main parameters used as indices of anxiety-related behaviour were: exploration preference for the novel compartment (relative to the familiar compartment), number of entries into the novel compartment and the latency of first entry to the novel compartment. These same parameters were also exclusively calculated for the distal part of the novel compartment. In order to assess the locomotor activity of subjects, the parameters; the total duration of movement, the average velocity of locomotion and the distance moved, were analysed for the whole apparatus.

4.2.3 Statistical analyses

The data were subjected to either one-way analysis of variance (ANOVA) for a genotype (between-subject) comparison of performance for the entire experimental session, or a two-way repeated measures ANOVA to examine the effect of genotype on performance of subjects, on minute-to-minute basis (i.e. minute blocks), as well as to investigate possible interaction across genotype groups in behaviour over time. Moreover, any interactions observed, were followed-up by an additional one-way ANOVA or repeated measures ANOVA between the individual levels of the within-subject factor, if or where required. Any significant difference between genotypes on parameters of anxiety-related behaviours, across behavioural batteries and within cohort, was followed-up by correlation analysis (Pearsons *r*). A one sample T-test comparative analysis was carried out to establish regional preference, relative to 50% rate. Finally, data from the separate WT-lines were clustered together for analysis where no significant differences between the two groups were observed (see Appendix II), in order to increase the statistical power and for reasons mentioned previously (in Chapter II, section 2.4.7).

4.3 Results

4.3.1 The elevated plus maze paradigm

Analysis of the data collected from the digital video tracking system showed that, as expected, subjects of all genotypes spent a significantly greater time during the EPM session in the protected closed arms and the middle area (~70% of total test time, see figure 4.1) than on the exposed open arms; *Igf2*-P0 KO mice (one-sample T-test): $t(18)= 15.33$, $p<0.001$, $r= 0.96$; *Igf2*-Null KO mice: $t(13)= 10.43$, $p<0.001$, $r= 0.95$; WT controls: $t(43)= 15.01$, $p<0.001$, $r= 0.70$, and a within-subject analysis indicated a greater amount time spent on the open arms at both the onset of the experimental session and towards the end of the session, $F_{9,657}= 4.22$, $p<0.01$ (see Figure 4.1A). Moreover, KO mice of the *Igf2*-P0 line showed a significantly lower amount of time on the exposed open arms relative to the enclosed arms (one-way ANOVA; $F_{2,73}= 5.71$, $p<0.01$) in comparison to KO mice of the *Igf2*-Null line and WT controls (*post hoc* tests; $F_{1,30}= 6.84$, $p<0.05$ and $F_{1,61}= 10.27$, $p<0.01$, respectively), as shown in Figure 4.1B. There was a slight reduction in the number of entries onto the exposed open arms as the session progressed (Figure 4.2A, within-subject test: $F_{7,512}= 5.68$, $p<0.001$). Although the *Igf2*-P0 KO mice made on average fewer entries onto the open arms during the EPM session than subjects of other genotype groups (Figure 4.2B), this was not found to be significant (one-way

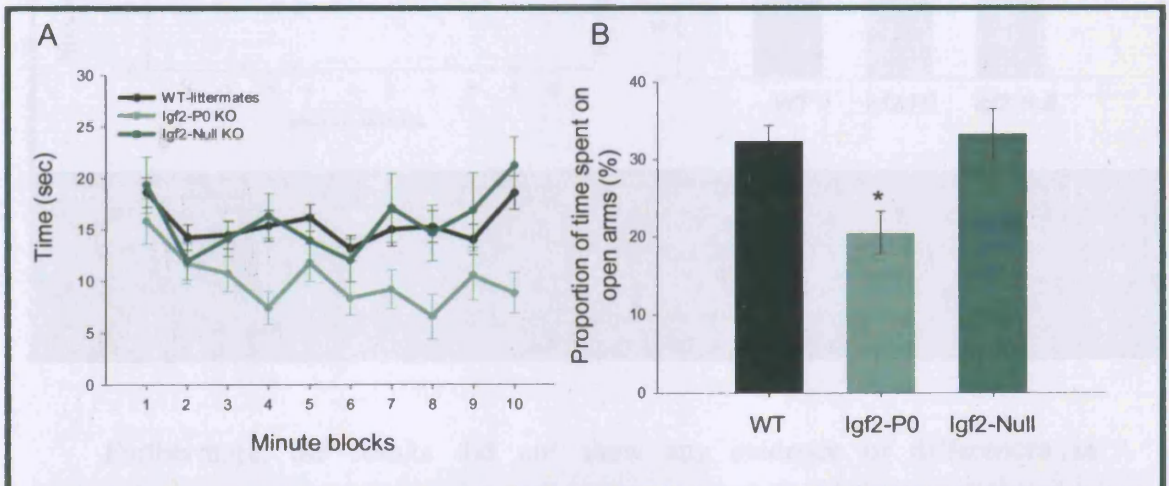

Figure 4.1: *Time spent on the open arms during the EPM.* ***(A)*** *The total amount of time spent on open arms between subjects of different genotypes, over the course of the 10 minute session.* ***(B)*** *The percentage of time spent on the open arms, between genotypes. Data are presented as mean values along with SEM.*

**Significant at $p<0.05$.*

ANOVA; $F_{2,73}$= 1.61, n.s.).

No difference was found between subjects of different genotypes for the latency of the first entry onto the exposed open arms (*Igf2*-P0 KO: 15.0 ± 4.8; *Igf2*-Null KO; 12.1 ± 1.8; WT controls: 10.0 ± 1.4, $F_{2,65}$= 1.04, n.s., one-way ANOVA; $F_{2,73}$= 1.01, n.s.). While a one-way ANOVA reported a significant difference between groups of different genotype in the number of rears (one-way ANOVA; $F_{2,65}$= 3.35, p<0.05), suggestive of fewer rearing among the *Igf2*-P0 KO mice (*Igf2*-P0 KO: 153.1 ± 18.2; *Igf2*-Null KO; 220.6 ± 29.5; WT controls: 204.2 ± 14.4), a *post hoc* comparison did not show this difference to be significant ($F_{2,73}$= 5.41, n.s.). No significant differences were found in either the number of head dips (*Igf2*-P0 KO: 56.4 ± 6.4; *Igf2*-Null KO; 71.1 ± 4.5; WT controls: 67.2 ± 3.6, $F_{2,65}$= 1.04, n.s.) nor stretch attends (*Igf2*-P0 KO: 22.6 ± 5.4; *Igf2*-Null KO; 23.9 ± 3.7; WT controls: 25.0 ± 1.3, one-way ANOVA; $F_{2,65}$= 1.04, n.s. and $F_{2,65}$= 0.20, n.s., respectfully) between genotypes.

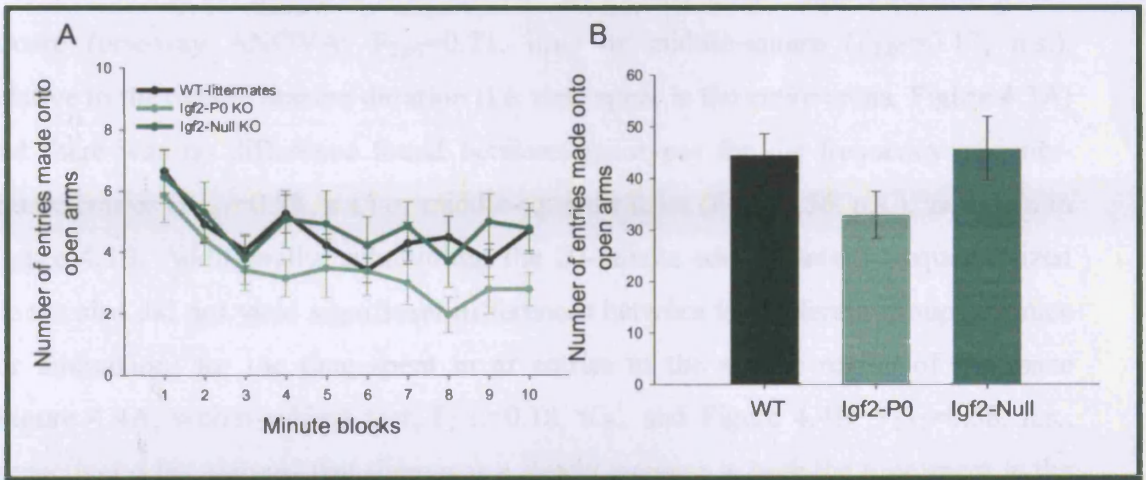

Figure 4.2: *Number of entries made into the open arms during the EPM.* ***(A)*** *The frequency of entries made onto the open arms between subjects of different genotypes, over the course of the 10 minute session.* ***(B)*** *The total frequency of entries onto open arms, between subjects of different genotype. Data are presented as mean values along with SEM.*

Furthermore, the results did not show any evidence of differences in locomotor activity between different genotypes. The total distance moved and time spent moving on the maze, during the session was equivalent across subjects (one-way ANOVA; $F_{2,73}$= 0.01, n.s. and $F_{2,73}$= 2.54, n.s., respectively) and the averaged velocity (cm/sec) of locomotion was also not significantly different ($F_{2,73}$= 0.01, n.s.).

A separate analysis of behavioural parameters between the WT controls of the different KO lines did not reveal any overall difference in the amount of time spent on the open arms or in the frequency of open arm entries, the WT littermates of the *Igf2*-Null KO line displayed elevated levels of activity on all three indices of locomotion (see Appendix 2.1).

4.3.2 The open field paradigm

The area of the OF was subdivided into three virtual regions using the Noldus video tracking software; outer, middle and inner. All of the subjects demonstrated thigmotaxic behaviour, concentrating the majority of their ambulation in the peripheral (outer) region near the walls of the OF, in contrast to the central region composed of the middle-square; *Igf2*-P0 KO mice (one-sample T-test): $t(16)=-22.95$, $p<0.001$, $r=0.99$; *Igf2*-Null KO mice: $t(10)=-22.82$, $p<0.001$, $r=0.99$; WT controls: $t(41)=-47.93$, $p<0.001$, $r=0.99$. Overall, there were no differences observed between subjects of different genotype in the percentage of time spent within the either inner-square (one-way ANOVA; $F_{2,67}=0.21$, n.s.) or middle-square ($F_{2,67}=0.17$, n.s.), relative to the overall session duration (i.e. time spent in the entire arena, Figure 4.3A) and there was no difference found between genotypes for the frequency of inner-square entries ($F_{2,67}=0.88$, n.s.) or middle-square entries ($F_{2,67}=0.56$, n.s.), as shown in Figure 4.3B. Additionally, subdividing the 20-minute session into 10 equally sized blocks also did not yield significant differences between the different groups of mice (or interaction) for the time spent in or entries to the middle region of the maze (Figure 4.4A, within-subject test; $F_{2,67}=0.18$, n.s., and Figure 4.4B, $F_{2,67}=0.58$, n.s., respectively) but showed that there was a steady increase in both the time spent in the central region (i.e. the inner-square) (Figure 4.4A, $F_{6,390}=10.92$, $p<0.001$) and the frequency of entries into the more central regions of the maze (Figure 4.4B, $F_{6,420}=13.77$, $p<0.001$), as the experiment progressed during the first 8-10 minutes. However, the number of entries into the central region began to slowly decline towards the latter half of the experimental session.

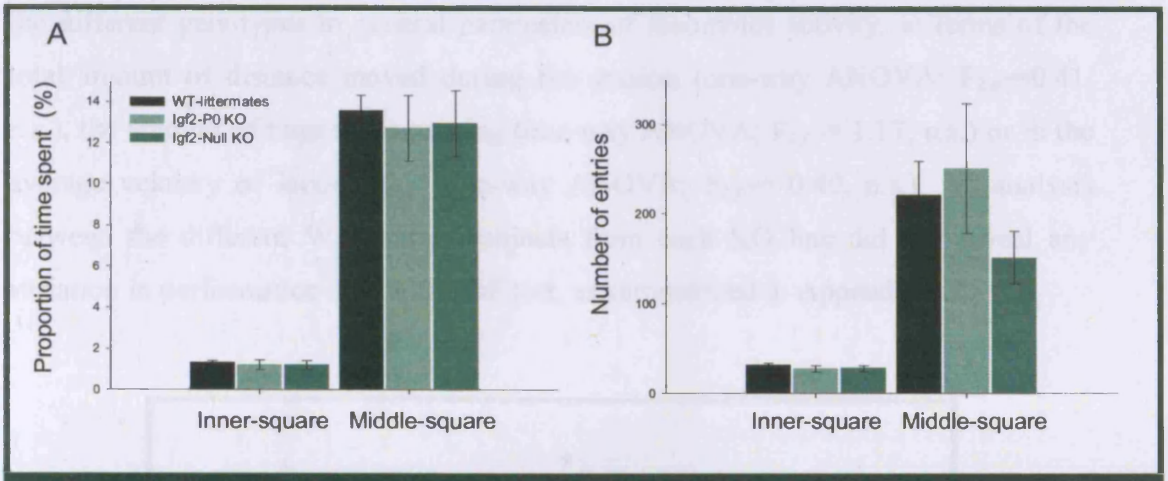

Figure 4.3: *Two of the main indices of anxiety-related behaviour during an OF session.* ***(A)*** *The percentage of time spent in the inner- and middle square relative to time spent in the entire arena, between subjects of different genotype.* ***(B)*** *Frequency of entries into the inner-square and middle square, between subjects of different genotype. Data are presented as mean values along with SEM.*

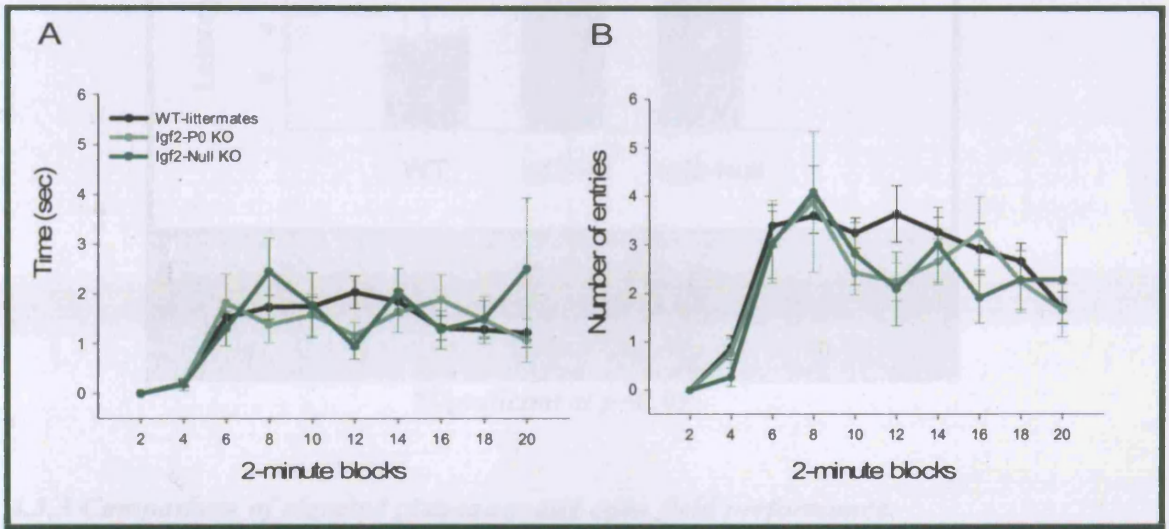

Figure 4.4: *Two of the main indices of anxiety-related behaviour across 2-minute blocks throughout the OF session.* ***(A)*** *The time spent in the inner-square, between subjects of different genotype.* ***(B)*** *Frequency of entries into the inner square, between subjects of different genotype. Data are presented as mean values along with SEM.*

Mice of the *Igf2*-P0 KO line displayed a significantly greater latency to enter the inner-square for the first time (Figure 4.5, one-way ANOVA; $F_{2,67}= 4.31$, $p<0.05$), relative to WT-littermate controls (*post hoc* test; $F_{1,57}= 8.10$, $p<0.05$), but this difference was not signififcant in comparison with *Igf2*-Null KO mice (*post hoc* test; $F_{1,26}= 4.46$, n.s.) and between *Igf2*-Null KO mice and the WT controls (*post hoc* test; $F_{1,51}= 0.00$, n.s.). In addition, there were no significant differences between mice of

the different genotypes in general parameters of locomotor activity, in terms of the total amount of distance moved during the session (one-way ANOVA; $F_{2,67}$=0.41, n.s.), the amount of time spent moving (one-way ANOVA; $F_{2,67}$= 1.17, n.s.) or in the average velocity of locomotion (one-way ANOVA; $F_{2,67}$= 0.40, n.s.). An analysis between the different WT-control subjects from each KO line did not reveal any variation in performance during the OF test, as summarized in Appendix 2.2.

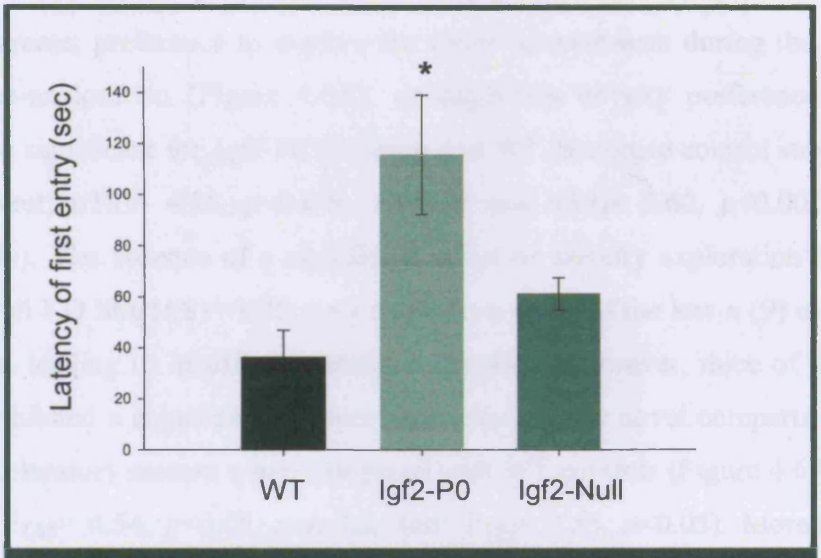

Figure 4.5: *The latency of first entry into the central region of the OF arena (inner-square), between subjects of different genotype. Data are presented as mean values along with SEM.*

**Significant at $p<0.05$.*

4.3.3 Comparison of elevated plus-maze and open field performance

A correlation analysis (pearson's *r*) was carried out on a subset of subjects that were included in the behavioural analysis in both the EPM and OF paradigms; *Igf2*-P0 KO mice (*n*): 17, *Igf2*-Null KO mice (*n*): 10 and WT controls (*n*): 37 (of which WT-P0 (*n*): 20 and WT-Null (*n*): 17). The results showed a moderate and significant negative relationship between percentage of time spent on the exposed open arm on the elevated plus maze and latency of first entry into the central region of the open field arena (inner-square), or r=-0.38, (one-tailed) $p<0.001$, respectively. Furthermore, when the data were examined separately for each genotype, the results showed that this association was only significant for mice of the *Igf2*-P0 KO line, at r= -0.52, p (one-tailed)<0.05 (*Igf2*-Null KO: r= -0.18, p (one-tailed)=n.s.; WT controls: r=-0.22,

p (one-tailed)= n.s.). Therefore, this significant correlation between separate measures of behaviour from two independent paradigms further supports the idea of increased levels of anxiety in the *Igf2*-P0 KO line of mice.

4.3.4 The free-exploratory paradigm

A second group of mice were assessed for performance in the FEP, where exploration between habituated and novel compartments was compared. All subjects exhibited greater preference to explore the novel compartment during the 15 minute test of free-exploration (Figure 4.6A), although this novelty preference was only found to be significant for *Igf2*-P0 KO mice and WT-littermate control subjects (one-sample T-test; $t(18)= 4.25$, $p<0.001$, $r= 0.99$ and $t(39)= 7.60$, $p<0.001$, $r= 0.99$, respectively). The absence of a significant effect on novelty exploration by mice of the *Igf2*-Null KO line ($t(8)= 1.75$, n.s.) might be a result of the low *n* (9) in this group and, hence, leading to insufficient statistical power. However, mice of the *Igf2*-P0 KO line exhibited a significantly lower preference for the novel compartment during the free-exploratory session when compared with WT controls (Figure 4.6A, one-way ANOVA; $F_{2,65}= 4.54$, $p<0.05$, *post hoc* test: $F_{1,57}= 7.55$, $p<0.05$). Moreover, while there was no difference observed in latency of first entry into the novel compartment across subjects (one-way ANOVA; $F_{2,65}= 0.06$, n.s.), the latency to enter distal part of the novel compartment was significantly increased among *Igf2*-P0 KO mice (Figure 4.6B, one-way ANOVA; $F_{2,65}= 4.20$, $p<0.05$, *post hoc* test: $F_{1,57}= 7.75$, $p<0.05$).

Conversely, there was no difference found in the proportion of time spent in the distal part of the novel compartment between subjects of the different genotypes (one-way ANOVA; $F_{2,65}= 0.24$, n.s.), the number of entries made into the novel compartment (one-way ANOVA; $F_{2,65}= 0.82$, n.s.) or into the distal part of the novel compartment (one-way ANOVA; $F_{2,65}= 2.21$, n.s.). As expected, a repeated measures analysis indicated that the number of entries into the novel compartment declined rapidly for all subjects as the experimental session progressed (Figure 4.7A, within-subject test; $F_{9,585}= 26.98$, $p<0.001$), whereas preference for the novel compartment remained relatively stable across the session (Figure 4.7B, within-subject test; $F_{5,304}= 1.77$, n.s.). There were no significant effects of the different genotypes on measures of locomotor performance during the exploratory session in terms of distance moved, as reported by a one-way ANOVA ($F_{2,65}= 1.96$, n.s.), the amount of time spent moving

($F_{2,65}$= 1.46, n.s.) or the average velocity of locomotion ($F_{2,65}$= 0.90, n.s.). Also, no difference was observed between WT controls of the different mutant lines, as shown in Appendix 2.3.

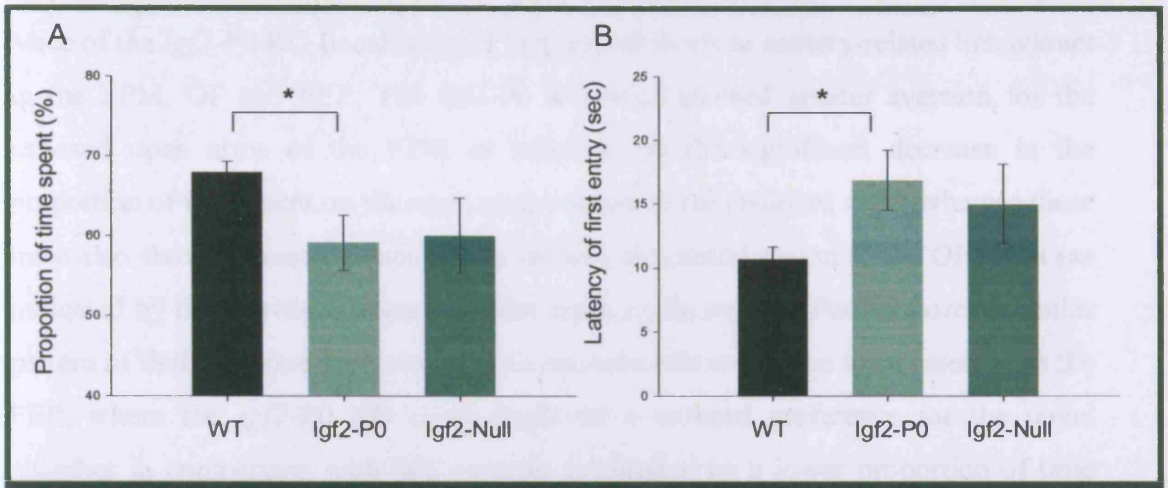

Figure 4.6: *Behavioural measures in the FEP.* ***(A)*** *The percentage of time spent in the novel compartment relative to time spent in the familiar compartment, across subjects of different genotype.* ***(B)*** *Latency of first entry into the distal part of the novel compartment, between subjects of different genotype. Data are presented as mean values along with SEM.*

**Significant at $p<0.05$.*

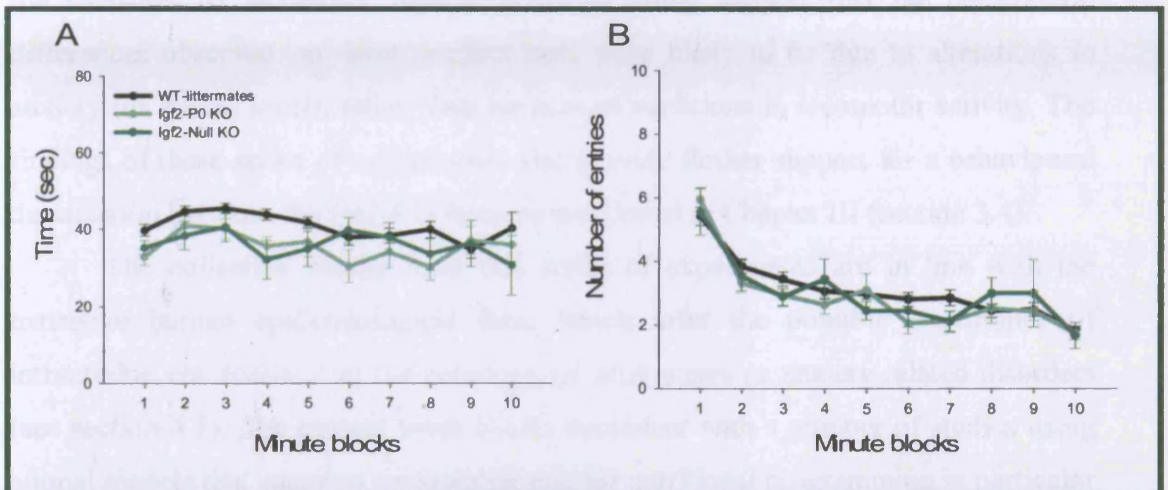

Figure 4.7: *Two of the main indices of anxiety-related behaviour across 2-minute blocks throughout the FEP session.* ***(A)*** *The time spent in the novel compartment, between subjects of different genotype.* ***(B)*** *Frequency of entries into the novel compartment, between subjects of different genotype. Data are presented as mean values along with SEM.*

4.4. Discussion

The converging findings of this series of experiments provide evidence for an enduring alteration in stress and anxiety-related behaviour, as a consequence of IUGR by placental insufficiency, and builds upon the results found in the previous chapter. Mice of the *Igf2*-P0 KO line displayed heightened levels in anxiety-related behaviours in the EPM, OF and FEP. The *Igf2*-P0 KO mice showed greater aversion for the exposed open arms of the EPM as reflected by the significant decrease in the proportion of time spent on the open arms relative to the enclosed arms, whereas these mice also showed greater reluctance to explore the central region of the OF arena (as indicated by the increased latency of first entry to the region). Furthermore, a similar pattern of findings were produced when a separate cohort of mice were assessed in the FEP, where the *Igf2*-P0 KO mice displayed a reduced preference for the novel chamber in comparison with WT controls (suggested by a lower proportion of time spent in the novel compartment) and showed greater reluctance to explore the distal part of the novel compartment. Mice of the *Igf2*-Null KO line did not show any significant difference on any of the conflict tests of anxiety-related behaviour. Moreover, the fact that the *Igf2*-P0 KO mice did not exhibit any differences on any of the measures of locomotor aspects provides strong support that the behavioural differences observed on these conflict tests were likely to be due to alterations in anxiety (or stress) levels, rather than because of variations in locomotor activity. The findings of these series of experiments also provide further support for a behavioural dissociation between the *Igf2*-KO lines, as mentioned in Chapter III (section 3.4).

The collective results from this series of experiments are in line with the extensive human epidemiological data, which infer the possible importance of intrauterine environment in the aetiology of adult stress or anxiety-related disorders (see section 4.1). The present work is also consistent with a number of studies using animal models that suggests an essential role for nutritional programming in particular (see section 4.1). Moreover, these data may be of relevance to research examining the effects of IUGR on the hypothalamic-adrenal-pituitary (HPA) axis and appears to be consistent with the evidence of later anxiogenic consequences of intrauterine malnutrition, which has emerged from the human and animal work (see Chapter I, section 1.4.2.1 for review). In fact, hyperactivation of the HPA axis, implicitly linked via the actions of cortisol in stress responses and anxiety (see Weber, 1998 for

review), has also been proposed as the nexus in mediating early life adversities with metabolic and cardiovascular sequelae in later life (McMillen and Robinson, 2005). Several studies have demonstrated raised levels of plasma cortisol in adulthood, among individuals of LBW (Lewitt, et. al., 2000; Philips et al., 2000; Philips et al., 1998) or born SGA (Cianfarani et al., 2002; Houang et al., 1999). Consistent with the human studies, exposure of foetal rats to maternal under-nutrition has also been shown to result in elevated plasma corticosterone levels during early postnatal life (Navarrette et al., 2007; Lesage et al., 2001). Similarly, plasma corticoid levels were found to be elevated in lambs born to ewes that had been briefly exposed to moderate under-nutrition during late gestation (Edwards and McMillen, 2001), although opposing effects have also been observed with more prolonged nutritional restrictions in sheep (Bispham et al., 2003). In this context, the current findings highlight the importance of investigating HPA functionality in *Igf2*-P0 KO mice (see section 8.7).

The limbic system (incorporating the amygdala and hippocampus) has been implicated as one of the key neuroanatomical substrates underlying anxiety and fear related behaviours (Engin and Dallas, 2007; Bannerman, et al., 2004). Further, while amygdala lesions have been shown to distinctively impact upon the fear response during fear-conditioning tasks, lesions to the hippocampus are noted to particularly exert effects on unconditioned ethologically based tests of anxiety (see Bannerman et al., 2004 for review) such as those presented here. For instance, a marked reduction in anxiety-related behaviour was observed in tests of social interaction and the successive allays apparatus (a modified version of the EPM), following selective lesions to either dorsal or ventral parts of the hippocampus, whereas ventral hippocampal lesions have also been found to result in anxiolytic effects on the EPM and light-dark test (McHugh, Deacon, Rawlins and Bannerman, 2004; Kjelstrup et al., 2002). Similar anxiolytic effects have been reported, when preceded by electrical stimulation of the hippocampus (Dringenberg, Levine and Mernard, 2008). Moreover, pronounced over-secretions of adrenocortioctrophine and corticosterone have been noted among rats in response to acute restraint stress, as well as heightened basal corticosterone levels, following hippocampal lesions (Feldman and Weidenfeld, 1995).

Although a number of neurotransmitter systems have been implicated in the control of stress and anxiety-related behaviour (e.g. cholesystokinin, noradrenaline and glutamate), the GABA- and serotonergic (5-HTergic) systems are generally

thought to have the major role (Millan, 2003; Clement and Chapouthier, 1998). Moreover, it is notable that the anxiety-modulating receptor subtypes of the GABA- and serotonergic systems have enriched expression in the hippocampus (Mehta and Ticku, 1999; Hoeyer et al., 1994, respectively). A number of micro-infusion studies have demonstrated a reduction in anxiety-related behaviour on various conflict tests of anxiety, following hippocampal injections of direct or indirect benzodiazepine acting $GABA_A$ receptor agonists (Rezayat, RoohBakhsh, Zarrindast, Massoudi and Djahanguiri, 2005; Menard and Treit, 2001; Gonzalez, Ouagazzal and File, 1998). On the other hand, the functional role of the 5-HT system in stress- and anxiety-related behaviour is less clear, where some studies have reported intra-hippocampal injection of 5-HT_{1A} agonists to have anxiolytic effects on the elevated plus maze (Menard and Treit, 1998; Kotwoski, Plaznik and Stefanski, 1989) or no differences on anxiety-related performance (Files, Gonzalez and Andrews, 1996; Belcheva, Belcheva, Petkov and Petkov, 1994). Moreover, an anxiogenic effect of 5-HT_{1A} agonists on subsequent testing of social interaction has even been recorded (Andrew, Hoggs, Gonzalez and File, 1994), with a reduction in anxiogenic behaviour on the elevated T-maze, following injection of SR46349B, a selective 5-HT_{2A} antagonist (Mora, Netto and Graeff, 1997). Unfortunately, the reasons for these paradoxical findings are not entirely certain but they still highlight a significant involvement of hippocampal 5-HT systems in modulating stress- and anxiety-related behaviours.

Therefore, the early developmental vulnerability of the hippocampus, illustrated in previous research (see Chapter I for review), may suggest this neural circuitry plays a role in the mediation of the anxiety-related phenotypes observed among the *Igf2*-P0 KO mice. Further, the results from the aforementioned neurophysiological investigations of stress and anxiety-related disorders, also suggest that either GABA- and/or 5-HTergic neurotransmitter pathways might be affected by altered developmental trajectories from the early life nutritional insult. In order to provide further evidence for this possibility, a neurobiological assessment of the *Igf2*-P0 KO mice in relations to their WT-littermates, is required (see Chapter VIII).

4.4.1 Summary of key results from Chapter IV

- *Igf2*-P0 KO mice spent proportionally less time on the open arms of the EPM, relative to *Igf2*-Null KO mice and WT controls.
- *Igf2*-P0 KO mice displayed greater latency to enter the exposed open region of the OF arena, than *Igf2*-Null KO mice and WT controls.
- In the FEP, the *Igf2*-P0 KO spent significantly less time in the novel compartment of test apparatus and showed greater latency to enter the distal part of the novel compartment, when compared to *Igf2*-Null KO mice and WT controls.
- *Igf2*-Null KO did not show any difference from WT controls on any of the conflict tests of anxiety.
- No difference was found between the *Igf2*-KO mice and WT controls, on any of the parameters indexing locomotor competence, during the EPM, OF and FEP.

Chapter V

Investigating Attentional Function and Behavioural Inhibition in Igf2-P0 and Igf2-Null Knockout lines: 5-Choice Serial Reaction Time Task

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5.1 Introduction

Attention deficit-hyperactivity disorder (ADHD, DSM-IV-TR, 2000) is one of the many neuropsychiatric disorders that have been associated with intrauterine life events (Breslau, 1995). ADHD is an early-onset pathophysiologically heterogeneous disorder, highlighted by poor sustained attention, hyperactivity and impulsivity. It is one of the most commonly diagnosed behavioural disorders in children and adolescents, with prevalence varying between 5-10% of children (Linnet et al., 2003), and diagnosis being approximately four times more common in males than females (Cuffe, Moore and McKeown, 2005). Findings from family-gene, twin, adoption and segregation analysis studies indicate that ADHD is a highly heritable neuropsychiatric disorder (see Franke, Neale and Faraone, 2009 for review). However, despite extensive efforts from recent candidate-gene association and genome wide linkage research, no major genes have been identified for ADHD (main candidate genes explain about 1% of the variance of phenotypes), and individual gene variants have only displayed modest effects (Plomp, Engerland and Durston, 2009). Moreover, according to data from monozygous twin studies the concordance of ADHD is estimated to be about 0.76 (Faraone and Biederman, 2005; Thapar, Holmes, Poulton and Harrington, 1999), which suggests that the aetiology of ADHD is also likely to include environmental factors or perhaps more likely, as a result from the interplay between genetic and environmental risk factors.

In the last decade, there has been an increased interest in the notion that ADHD susceptibility could be partially related to a suboptimal foetal environment. In this regard, a number of case-control studies have consistently reported higher rates of hyperactivity and inattention among children of low birth weight (LBW) in comparison to normal birth weight children, based on assessments by parents and teachers (Indredavik, Heyerdahl, Kulseng and Brubakk, 2005; Zubrick et al., 2000:

Breslau et al., 1996; McCormick, Workman-Daniels and Brooks-Gunn, 1996). Moreover, children born at term with birth weights ranging between 1500-2499g had a 90% increased risk of being diagnosed with ADHD (Linnet et al., 2006), but in parallel, children with ADHD were shown to be three times more likely to have been born with LBW relative to nonADHD children (Mick, Biederman, Faraone, Sayer and Kleinman, 2002). Similar findings have also been demonstrated in studies where weight and size at birth were adjusted for gestational age (Schlotz, Jones, Godfrey and Philips, 2008; Lahti et al., 2006; Indredavik et al, 2005).

Although these retrospective epidemiological studies support the notion that part of the susceptibility for ADHD can be attributed to early life events, the exact implications of these findings are difficult to interpret. One issue is that foetal weight and size (even in at-term births) do not necessarily represent surrogate measures for foetal growth. Another issue concerns the fact that birth weight and size is a consequence of many factors, both genetic and environmental. Some of these environmental factors may be related to ADHD susceptibility, whereas others may not. Alternatively, intrauterine growth retardation (IUGR) *per se* might affect ADHD susceptibility, regardless of the nature of its causal determinants. While suboptimal nutrition supply to the growing foetus has been advocated as one of the key programming stimuli that underlie the relationship between IUGR and later life disorders (Gallou-Kabani and Junien, 2005; Harding, 2004; Barker, 1997), other environmental factors such as maternal alcohol use, smoking and stress, have also been implicated (see Kramer, 1987 for review). In fact, in previous epidemiological studies, ADHD symptoms have been positively correlated with maternal alcohol use (Mick et al., 2002; Streissguth, Sampson and Barr, 1989), smoking (Mick et al., 2002; Milberger, Biederman, Faraone and Jones, 1998) and stress (O'Connor, Heron, Golding, Beveridge and Glover, 2002; McIntosh, Mulkins, and Dean, 1995), independently of birth weight; importantly, there have not been any studies showing such relationship between intrauterine malnutrition and ADHD susceptibility.

Therefore, the main objective of this chapter is to investigate whether intrauterine malnutrition and foetal growth retardation in genetic model system, affect attentional function and impulsivity (in terms of response inhibition), in later life. In this study, aspects of attentional function and impulsivity were compared between mice of both *Igf2*-KO lines and wild-type (WT) littermates, using the 5-choice serial reaction time task (5-CSRTT). The 5-CSRTT is a widely used test of attentional

performance in rodents (Higgins and Breysse, 2008), and has been considered to be an analogue of the continuous performance task (CPT), which examines attentional functions in humans (Robbins, 2002). In the 5-CSRTT, subjects are required to respond as accurately and as rapidly as possible to a brief visual stimulus which is randomly presented in one of five spatial locations, in order to receive a reinforcer. Since accurate responding requires attention on both temporal and spatial domains by the subject, this task provides considerable parametric flexibility and potential independent assessment of the spatial and temporal component of attention (e.g. between selective and sustained attention) (Bushnell, 1998). Furthermore, although discriminative accuracy performance can be confounded by other sensory, motor or motivational processes, these interpretations can be disambiguated to some extent, by considering the overall behavioural profile of the results (see Robbins, 2002 for review). While the 5-CSRTT was primarily developed to examine attentional functions in rodents, aspects of response inhibitory control are also indexed by the number of premature responses, occurring inappropriately during the interval between trials and prior to the presentation of the visual stimulus. Other measurement of behavioural phenotypes provided by the task, include; motivation (latency to collect the reinforcer , the effects of *ad libitum* feeding prior to test, number of trials and reaction times) and locomotor activity (as indexed by beam-breaks crossing the operant chamber and reaction times). Moreover, a number of task manipulations may be introduced during the course of this task, in order to tax dissociable aspects of behaviour.

5.2 Materials and Methods

Full methodological descriptions can be found in the relevant sub-sections of the General Methods Chapter (Chapter II).

5.2.1 Subjects and animal husbandry

A cohort of 95 male mice (generated from the Behavioural Neuroscience Laboratory at Cardiff University), aged 13-16 weeks at the onset of experiment, were subjected to testing on the 5-CSRTT (see Table 5.1). The mice were obtained from a crossbreeding of background strain of C57BL/6*129 (carrying either the mutant *Igf2*-

P0 allele or the *Igf2*-Null allele) with CD1 female, as previously described in Chapter II, section 2.1.

Table 5.1: *The sample size and genotype of the total litter cohort which began and completed the 5-CSRTT.*

Status	Genotype and sample size (*n*)		
	Igf2-P0	*Igf2*-Null	WT (WT-P0/WT-Null)
Number of subjects to begin the 5-CSRTT	29	16	50 (30/20)
Number of subjects to complete the 5-CSRTT	22	13	37 (25/12)

Mice were housed in littermate groups of two to five animals per cage, under temperature- and humidity-controlled conditions, with a 12-hour light: 12-hour dark cycle (lights on at 07:30 hours). All subjects had access to standard laboratory chow and water *ad libitum*, unless otherwise mentioned. From the onset of the experiment, mice were maintained on a regime of regulated access to water in the home cage (as detailed in section 2.4.4). Initially, for 4-hours/day (for 2 days) before reduction to 2-hours/day for the duration of behavioural testing. Upon stabilization of body weight under this regime, mice began habituation of the reinforcer followed by testing on the 5-CSRTT. Throughout this period, body weight was monitored regularly, and any mice losing more than 10% of normal body weight (measured during *ad libitum* water) were returned to *ad libitum* water. All experimental procedures were conducted under licenses issued by the Home Office (U.K.) in compliance with the Animals (Scientific Procedures) Act 1986 and local ethics committee.

5.2.2 Behavioural methods

5.2.2.1 Reinforcer habituation and preference testing

Habituation to the condensed milk solution (10%) reinforcer was carried out for all subjects across six daily sessions of 10 minutes. For the first two days, mice were presented with two bowls of water during, while in the following four sessions the mice were presented with one bowl of water and one bowl of condensed milk solution. For each session, the volume of each liquid consumed was recorded and total liquid consumption, and condensed milk preference was calculated in terms of as the

condensed milk consumed/total volume of liquid consumed (see Chapter II, 2.4.5 for further details). Due to the weight differences between the *Igf2*-P0 KO, *Igf2*-Null KO and WT-control mice, the volumes consumed were normalised for body weight using Kleiber's 0.75 mass exponent (Schmidt-Nielsen, 1990).

5.2.2.2 5-choice serial reaction time task (5-CSRTT)

The experimental work for the 5-CSRTT task was carried out in nine-aperture operant chambers, previously modified for use in mice (Humby et al., 1999) with four of the nine apertures, apertures 2, 4, 6 and 8, in the response array blocked (see Chapter II, section 2.5.7). Aspects of attentional functions and impulsivity were examined by measurement of discriminative accuracy and the number of premature nose-pokes respectively, but other parameters of interest included; omissions, trial numbers, correct reaction time (correct RT), magazine latency (MagLat). The mice performed one 20-minute session per day with a maximum of 100 trials per session. See Chapter II, section 2.3 for further details.

5.2.2.2.1 Initial behavioural shaping

Shaping was carried out over eight consecutive daily sessions (of 20-minute duration each) in order to habituate the mice to the chambers and train them to obtain the condensed milk reinforcer from the food magazines. All of the apertures in the response array were covered with a transparent plastic film, throughout these shaping sessions. In the first three sessions, the door to the food magazine panel was wedged open to enable easy access to the reinforcer, and reinforcer delivery (50 deliveries of 40µl each, 30-second intervals) was accompanied by an illumination of the tray light in the food magazine. On the fourth and fifth sessions, the magazine panel was released which constrained the mice to push the panel open (termed 'panel-push') in order to gain access to the reinforcer. Any mice which failed to learn this procedure were given additional shaping sessions to overcome this obstacle. In the sessions seven and eight, the structure of the task was altered such that the delivery of the reinforcer was under the control of the subjects (i.e. by emitting a conditioned response). Thus, a panel push initiated the onset of a 10-second light stimulus (presented pseudo-randomly at each of the five stimulus positions in the nose poke

array) along with the delivery of the reinforcer 1 second prior to light stimulus offset, thus allowing the mice to associate the stimulus offset with delivery of the reinforcer. The tray light illuminated concomitantly with the reinforcer delivery and remained until the subject made another panel-push, initiating the next trial. The final two shaping sessions lasted for 20 minutes or upon reaching 100 trials (which ever occurred first), and apart from the nose-poke component, constituted the basic trial design of the 5-CSRTT.

5.2.2.2.2 5-CSRTT: trial design

From session nine, the transparent plastic film covering the apertures in the response array was removed allowing subjects to make nose poke responses to the stimuli. Each session lasted for 20 minutes or upon reaching 100 trials, which ever occurred first, and at the beginning of each session the first trial was initiated by a panel-push and commenced with a 5-second inter-trial interval (ITI), followed by a stimulus presentation in one of the five apertures of the response array. The sequence of the light stimulus presentation was predetermined by a pseudo-random schedule, by which there were four presentations at each of the five apertures within a block of 20 trials (thus five blocks within a session). Subjects were required to respond with a nose-poke in the illuminated aperture within the duration of the stimulus presentation and a 5-second limited hold period. If the mice emitted a response in the correct aperture within this time period, reinforcer (20µl condensed milk) was dispensed in the food magazine along with an illumination of the tray light (see Figure 5.1). The next trial was initiated by the collection of the reinforcer (i.e. panel-push) and the subsequent exiting of the food magazine.

Alternatively, if mice responded with a nose-poke in any of the four non-illuminated apertures (incorrect response) or failed to make a response within the defined response period (omission), a 5-second time-out period was implemented with the onset of the house lights. Similarly, a premature response during the ITI also resulted in a 5-second time-out period. However, the time-out period could be cancelled by a panel-push, which started a new trial.

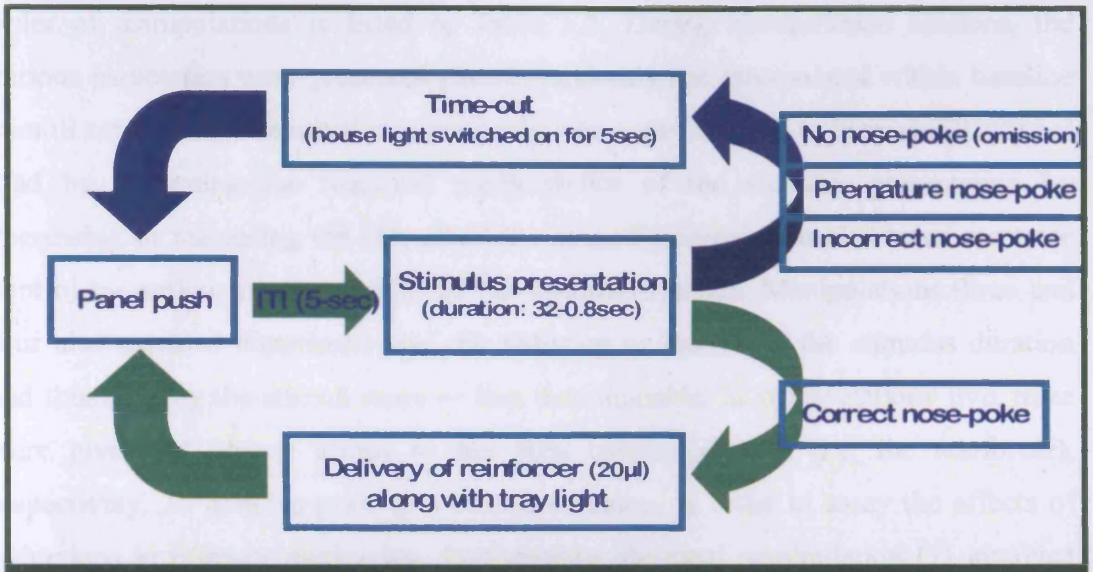

Figure 5.1: *Schemata of the possible sequences of events during a 5-CSRTT trial. Trials were initiated by a panel push, with an inter-trial interval (ITI, 5-sec at baseline) followed by brief stimulus presentation (0.8-sec at baseline) at one of five possible apertures in the nine-hole array. If subjects made a correct response, reinforcer is delivered in the food magazine behind the panel, and the next trial begins. Alternatively, if the subject fails to make a response, makes an incorrect response or responds too early, a 5-sec time-out period is signalled by the house light.*

5.2.2.2.3 5-CSRTT: training to baseline performance

At the outset of 5-CSRTT sessions, the length of the stimulus presentation was set at 32 seconds to facilitate learning, although this duration was gradually reduced to a baseline duration of 0.8 second in the following stages; 32 second, 16sec, 8sec, 4sec, 2sec, 1.8sec, 1.6sec, 1.4sec, 1.2sec, 1.0sec and 0.8sec. The stimulus duration was reduced for each individual subject on attaining pre-established performance criteria across three consecutive sessions, which was confined to; >80% accuracy, <20% omission and >20 trials per session. No other parameters were altered during the acquisition of baseline performance.

5.2.2.2.4 5-CSRTT: behavioural manipulations

Once subjects had reached stable baseline performance at a stimulus duration of 0.8 second, defined by the same criteria as described above, a variety of behavioural manipulations designed to measure dissociable aspects of attentional load, impulsivity and motivation, were undertaken. Each manipulation was implemented following two consecutive days of stable criterion performance. The

order of manipulations is listed in Table 5.2. During manipulation sessions, the various parameters were presented pseudo-randomly and interpolated within baseline stimuli conditions. The initial two manipulations were designed to increase attentional load by disrupting the temporal predictability of the stimulus presentation by decreasing or increasing the ITI, albeit the second manipulation also taxed response control by prolonging the period for the awaited stimulus. Manipulations three and four also assessed attentional load, by reducing or increasing the stimulus duration and thus making the stimuli more or less discriminable. In manipulations five, mice were given *ad libitum* access to the 10% condensed milk (i.e. the reinforcer), respectively, 20 minutes prior to a baseline session, in order to assay the effects of reductions in primary motivation. Furthermore, the final manipulation (7) involved the imposition of a short burst of a distracting white noise (100dB), of 0.5 second length, at varying times within the 5-second ITI prior to the stimulus onset, in order examine aspects of selective attention.

Table 5.2: *Manipulations of task parameters in the 5-CSRTT.*

Manipulation	Parameters	Main effect on behaviour
(1) Short inter-trial interval	ITI: 5sec (baseline), 4sec, 3sec, 2sec	Increase attentional load
(2) Long inter-trial interval	ITI: 5sec (baseline), 6sec, 7sec, 8sec	Increase attentional load Reduce impulse control
(3) Short stimulus duration	Stimulus Duration: 0.8sec (baseline), 0.6sec, 0.4sec, 0.2sec	Increase attentional load
(4) Long stimulus duration	Stimulus Duration: 0.8sec (baseline), 1.2sec, 1.6sec, 2sec	Reduce attentional load
(5) *Ad lib* condensed milk access 20min prior to test	None	Reduce motivation
(6) White noise distracter (100dB) for 0.5sec	Presentation of distractor: None (baseline), at beginning of trial, at 2.5sec into ITI, coincident with stimulus presentation	Increase attentional load

5.2.2.2.5 1-choice serial reaction time task (1-CSRTT)

Upon completion of the final manipulation session in the 5-CSRTT the task protocol was modified such that stimulus presentation was limited to the central aperture only as a further assessment of alteration in attentional load. Altering stimuli presentation in this way, thus tested the extent to which any effects on discriminative

accuracy in 5-CSRTT were related to attentional effects. During the 1-CSRTT, the ITI was randomly varied within a baseline session (duration 4-seccond, 4.5-sec, 5-sec and 5.5-sec) to add a degree of temporal unpredictability. This was done in effort to ensure that the mice were reacting to the actual stimulus presentation, rather than merely timing their responses. In other respects, all protocol parameters remained in the same settings as in the 5-CSRTT.

5.2.2.2.6 Behavioural measures

Task acquisition of the 5-CSRTT was determined by the number of sessions required for each subject to reach baseline performance within the limits of the criteria outlined in section 5.2.2.2.3, and the number of sessions taken at each training level (i.e. level of stimulus duration). A number of different behavioural measures were recorded for each session, in order to establish a relatively broad behavioural profile of the *Igf2*-KO lines. The primary indices of attentional function were the percentage of discriminative response accuracy (i.e. correct responses/(correct + incorrect responses)) and the percentage of omissions, (number of omitted trials/total completed trials) reflecting possible failures of detection and/or motivational or motor deficits. The number of premature nose-pokes was used as an index of response control and among other measures of interest were; total number of completed trials, correct RT (i.e. latency to respond to the correct aperture after stimulus presentation), latency to collect the reinforcer following a correct response, duration of eating (i.e. magazine latency, defined as the time the food magazine door (panel-push) was held open on the initial visit of the subject after a correct response), and the number of nose-pokes, panel-pushes and beam-breaks per trial.

5.2.3 Statistical analysis

The data were either subjected to one-way analysis of variance (ANOVA) for a genotype (between-subject) comparison of performance, or a two-way repeated measures ANOVA to examine the effects of stimulus duration levels and levels of behavioural manipulations (both defined as within-factors) on performance within genotypes, as well as possible interactions. Moreover, any interactions observed, were followed-up by an additional one-way ANOVA or repeated measures ANOVA between the individual levels of the within-subject factor. The genotype of subjects

was defined as between-subject factor throughout the entire analysis, whereas specific within-subject factors were used in each analysis. Thus for the reinforcer habituation the following factor was used: the number of habituation session during reinforcer habituation, for 5-CSRTT acquisition the training stage, (defined by the stimulus duration), and the level of manipulation within 5-CSRTT manipulation sessions. A repeated measures analysis was also carried out in order to compare performance between the 5-CSRTT and 1-CSRTT. Finally, data from the separate WT-lines were clustered together for analysis see Appendix III (unless otherwise stated) in order to increase the statistical power and for reasons previously mentioned (in Chapter II).

5.3. Results

5.3.1 Reinforcer habituation and preference testing

Analysis of the milk reinforcer preference and total volume consumption of the mice was only carried out on subjects that completed the 5-CSRTT (Figure 5.2). The preference of subjects for the condensed milk reinforcer increased rapidly throughout the four habituation sessions (Figure 5.2A, within-subject test; $F_{3,209}$= 159.47, p<0.001) with no significant differences between the different genotype groups (between-subject test; $F_{2,69}$= 1.35, n.s). By the final habituation session, mice of all three genotypes had displayed a strong preference (>85%) for the condensed milk reinforcer. Furthermore, there was a steady increase in the total volume of liquid consumed over the course of the reinforcer habituation among all subjects (see Figure 5.2B, within-subject test; $F_{4,270}$= 54.78, p<0.001). There were no differences in the total volumes of liquid consumed between the different genotypes of mice after the volume-consumption data had been corrected for differences in bodyweight ($F_{2,69}$= 2.06, n.s.). Comparison between the two WT-control mutant lines (i.e. WT-P0 littermates and WT-Null littermates) did not reveal any difference in the preference for the milk reinforcer or in the overall volume of liquid consumed (see Appendix 3.1).

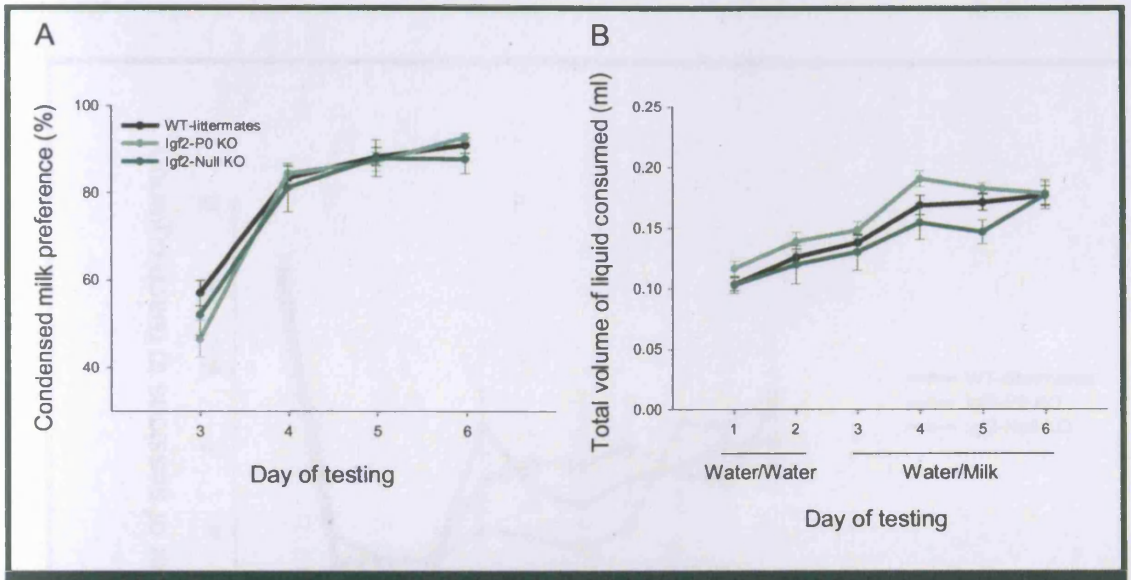

Figure 5.2: *Reinforcer habituation. Subjects were only presented with water during the first two days of reinforcer habituation, but in the following four days an option between water and condensed milk (10%) was present.* ***(A)*** *Subjects established a relatively strong preference for the condensed milk reinforcer by day four, reaching levels >85%.* ***(B)*** *Total liquid consumption (water and condensed milk combined), normalised for body weight of the subjects, gradually increased over the course of the reinforcer habituation sessions. For subject N see methods section 5.2.1. Data are presented as mean±SEM.*

5.3.2 5-CSRTT acquisition

Although, all of the subjects successfully acquired the 5-CSRTT procedure and reached baseline performance, only 76% of mice completed both 5-CSRTT and 1-CSRTT (including all the manipulations, see table 5.1). The total number of sessions required to reach stable baseline performance (~75 sessions) was equivalent between genotypes (one-way ANOVA; $F_{2,69}$= 0.63, n.s.) and there was no difference between the groups of mice in the number of sessions required to complete each stage of training (Figure 5.3, between-subject test; $F_{2,69}$= 1.30, n.s.). The large number of sessions required by the subjects to reach criteria at the 32-second stimulus duration (SD) level provided an index of the learning of the 5-CSRTT trial procedure and once having acquired the trial procedure, mice progressed relatively rapidly across the next few SD levels. However, once the stimulus duration decreased below 4 seconds the number sessions required to progress to subsequent levels, began to increase (within-subject test; $F_{6,410}$= 9.05, p<0.001). A separate analysis of WT-control lines (i.e. between WT-P0 and WT-Null control lines), did not show any differences in 5-CSRTT acquisition (one-way ANOVA; $F_{1,35}$= 0.37, n.s.).

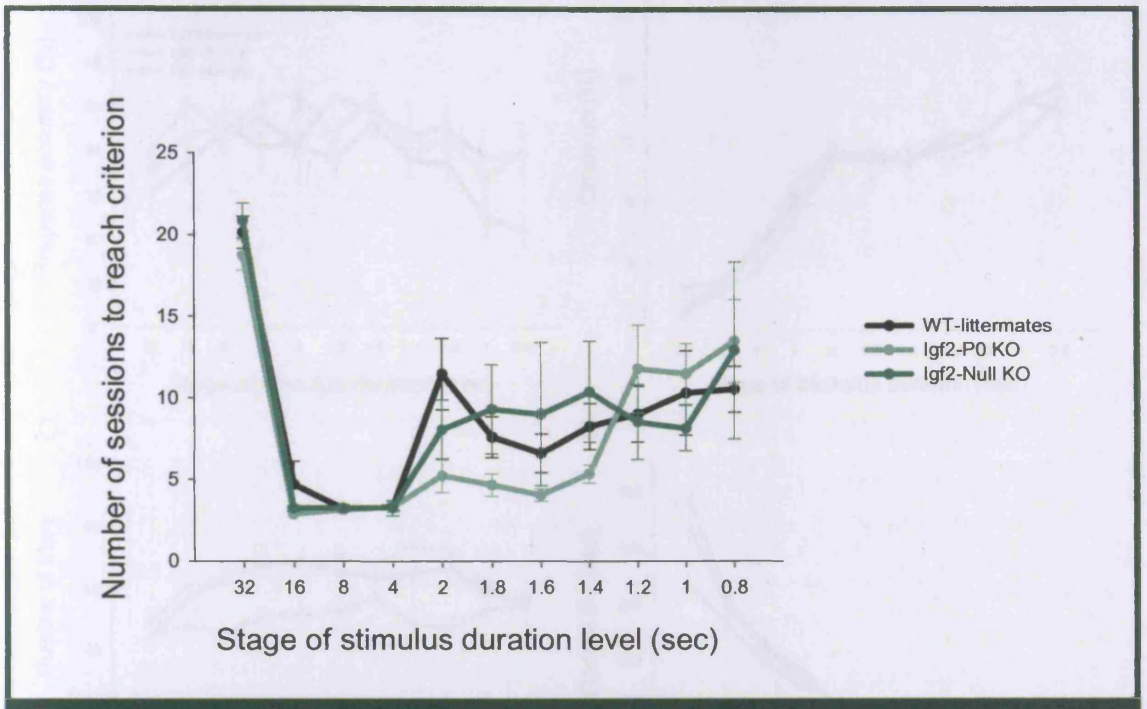

Figure 5.3: *5-CSRTT acquisition, in terms of the number of sessions that were required for subjects to reach criteria, at each of the stimulus duration (SD) levels. Data are presented as mean±SEM.*

The discriminative response accuracy of subjects showed a gradual overall increase during the 5-CSRTT acquisition phase (Figure 5.4A, within-subject test; $F_{8,560}$= 4.62, p<0.001), but began to decline as the stimulus duration approached the baseline (0.8-sec) level. More importantly, mice of the *Igf2*-P0 KO line displayed a significantly heightened discriminative accuracy during the acquisition phase relative to WT controls (between-subject test; $F_{2,69}$= 3.96, p<0.05; *post hoc* tests: $F_{1,57}$= 6.83, p<0.05), but no such difference was noted between *Igf2*-Null KO mice and WT-controls ($F_{1,48}$= 1.81, n.s.). The rate of omissions increased steadily among subjects as the SD level reduced during the acquisition phase, reflecting the rising attentional demands of the task (Figure 5.4B, within-subject test; $F_{6,448}$= 71.46, p<0.001), although the analysis did not reveal any difference across genotypes (between-subject test; $F_{2,69}$= 1.18, n.s.).

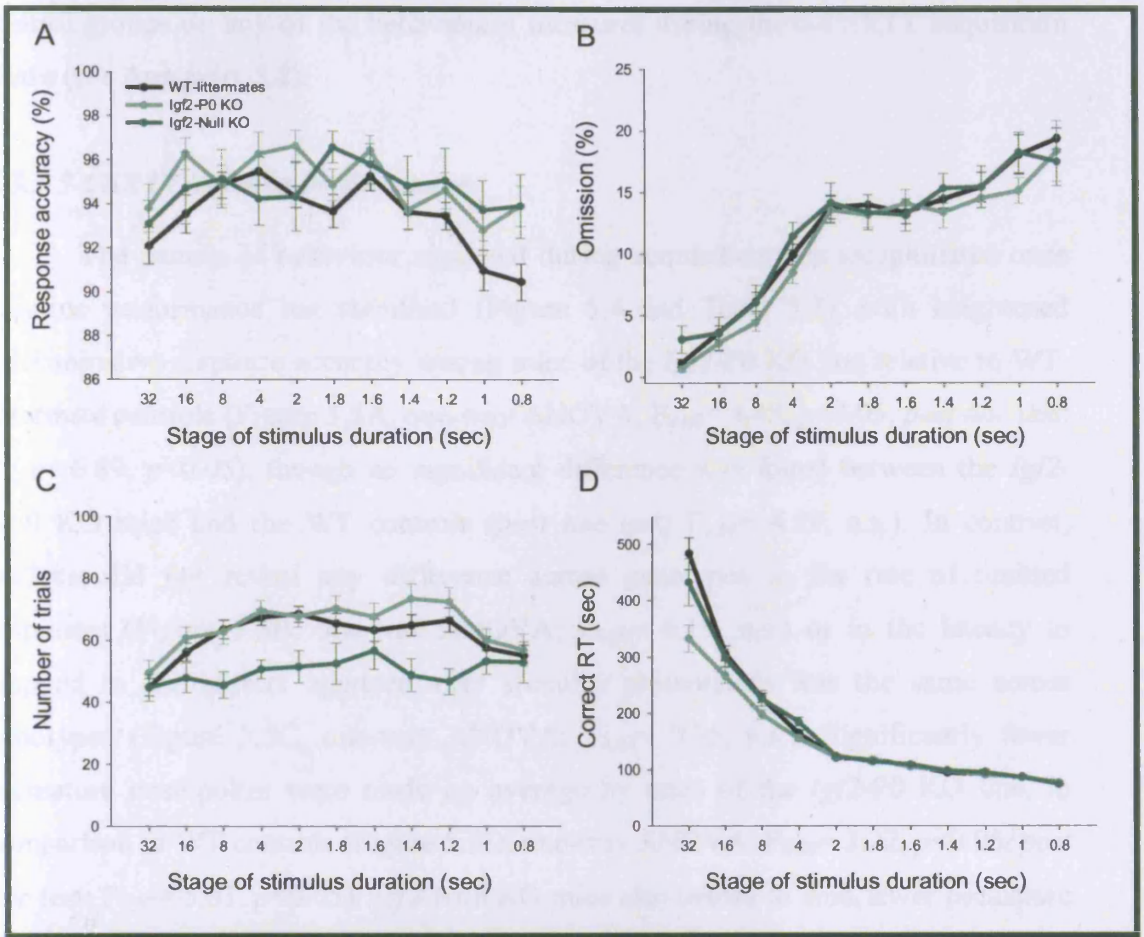

Figure 5.4: *Performance of subjects on major behavioural measures at the different SD levels during 5-CSRTT acquisition. Data taken from final session at each level, and shows response accuracy* ***(A)****, number of completed trials* ***(B)****, omitted trials* ***(C)*** *and correct response latency* ***(D)****. Data are presented as mean±SEM.*

Although the number of completed trials at each training stage was not consistent, *Igf2*-P0 KO and WT-control mice showed a significant increase in the number of trials through the middle stages of training (interaction between genotype and training stage, $F_{15,532}= 1.74$, $p<0.05$), but all groups of mice were performing an equivalent number or trials by the time the acquisition phase had been completed (Figure 5.4C, one-way ANOVA at SD of 0.8sec; $F_{2,69}= 0.61$, n.s.). Furthermore, the correct reaction time decreased during the course of acquisition (Figure 5.4D, within-subject test; $F_{2,119}= 255.60$, $p<0.001$) as the stimulus duration decreased. No main effect of genotype was observed (between-subject test; $F_{2,69}= 1.47$, n.s.) but there was a significant interaction between genotype group and training stage ($F_{3,119}= 5.44$, $p<0.001$) which indicated a lower correct RT among *Igf2*-P0 KO mice at the initial acquisition stage (i.e. SD= 32-sec). No difference was found between the two WT-

control groups on any of the behavioural measures during the 5-CSRTT acquisition phase (see Appendix 5.2).

5.3.3 5-CSRTT baseline performance

The pattern of behaviour observed during acquisition was recapitulated once baseline performance has stabilised (Figure 5.4 and Table 5.3), with heightened discriminative response accuracy among mice of the *Igf2*-P0 KO line relative to WT-littermate controls (Figure 5.5A, one-way ANOVA; $F_{2,69}$= 4.48, p<0.05, *post hoc* test: $F_{1,57}$= 6.89, p<0.05), though no significant difference was found between the *Igf2*-Null KO mice and the WT controls (*post hoc* test; $F_{1,48}$= 4.79, n.s.). In contrast, analysis did not reveal any difference across genotypes in the rate of omitted responses (Figure 5.5B, one-way ANOVA; $F_{2,69}$= 0.19, n.s.) or in the latency to respond to the correct aperture after stimulus presentation was the same across genotypes (Figure 5.5C, one-way ANOVA; $F_{2,69}$= 0.35, n.s.). Significantly fewer premature nose-pokes were made on average by mice of the *Igf2*-P0 KO line, in comparison to WT controls (Figure 5.5D, one-way ANOVA; $F_{2,69}$= 3.22, p<0.05, *post hoc* test: $F_{1,57}$= 5.61, p<0.05). *Igf2*-Null KO mice also tended to emit fewer premature responses than WT controls, albeit this difference did not yield significance (*post hoc* test: $F_{1,48}$=4.68, p= 0.085).

Assessment of other behavioural measures (see Table 5.3) showed that there was no evidence of difference in the number of trials initiated between genotypes (one-way ANOVA; $F_{2,69}$= 0.09, n.s.), however, *Igf2*-Null KO mice showed a considerably lengthened latency to collect the reinforcer following a correct response (Kruskall-Wallis test; H_2= 7.42, p<0.05), relative to *Igf2*-P0 KO mice (*post hoc* test: U= 73, p<0.05, r=-0.40) and WT controls (*post hoc* test: U= 147, p<0.05, r=-0.29). No genotype difference was found on other behavioural measures by one-way ANOVAs; duration of eating: $F_{2,69}$= 0.33, n.s., nose-pokes/trial: $F_{2,69}$= 1.92, n.s., and panel-pushes/trial: $F_{2,69}$= 0.07, n.s. A comparative analysis of the two WT-control lines did not show any difference on any the behavioural measures at baseline performance (see Appendix 3.3).

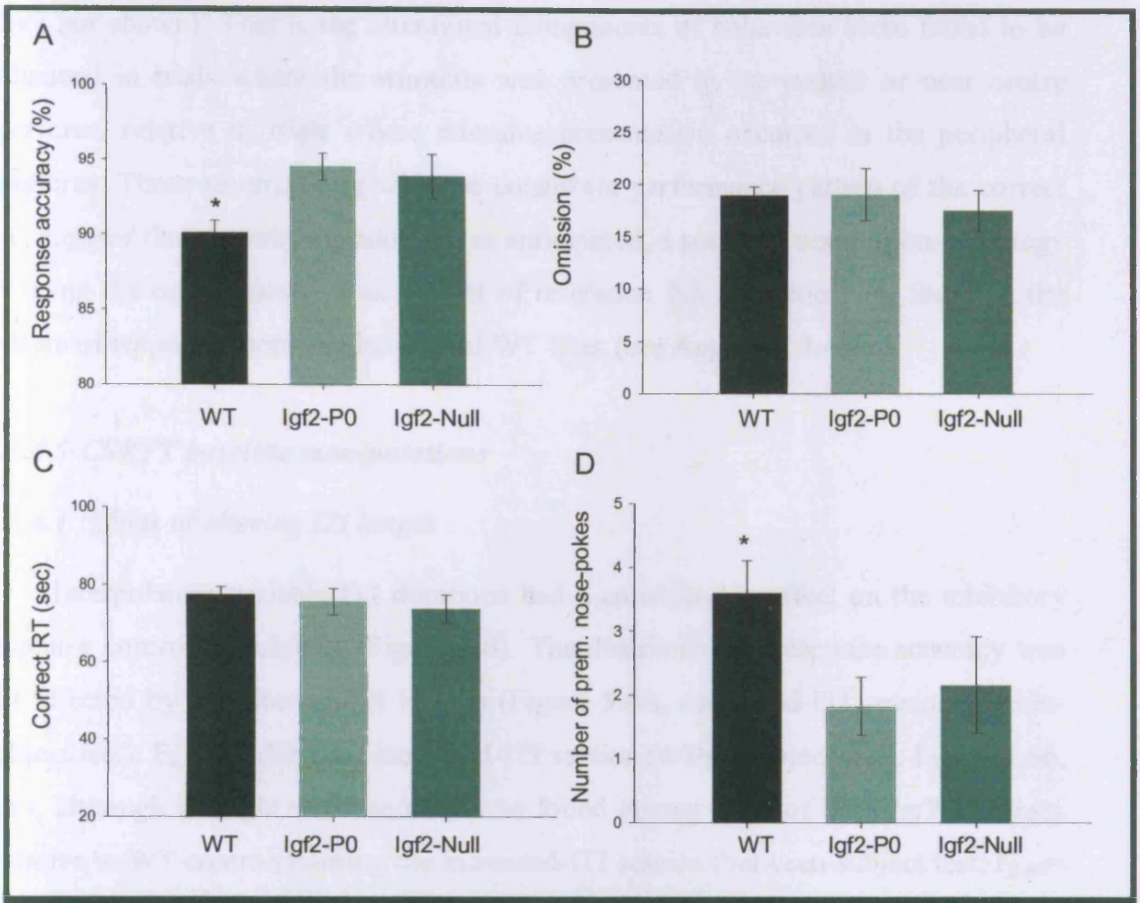

Figure 5.5: *5-CSRTT baseline performance. Response accuracy* ***(A)****, omitted trials* ***(B)****, correct reaction time* ***(C)*** *and the number of premature responses* ***(D)*** *from a baseline session following two consecutive sessions at criterion performance. Data are presented as mean±SEM.*

**Significant at $p<0.05$.*

Table 5.3: *5-CSRTT performance at baseline level following two consecutive baseline sessions at criterion performance.*

Behavioural Parameters	Mean ± SE		
	Igf2-P0	*Igf2*-Null	WT controls
Trial number	55.8 ± 5.1	52.8 ± 6.7	55.7 ± 3.4
Magazine latency (sec)	4.5 ± 2.8	*11.0 ± 4.0	2.2 ± 0.6
Duration of eating (sec)	2.5 ± 0.2	2.3 ± 0.5	2.6 ± 0.2
Premature nose-pokes	1.8 ± 0.5	2.2 ± 0.8	3.6 ± 0.5
Nose-pokes per trial	1.2 ± 0.1	1.3 ± 0.1	1.6 ± 0.2
Panel-pushes per trial	1.8 ± 0.2	1.6 ± 0.1	1.6 ± 0.1

**Significant at $p<0.05$.*

When the data were sub-divided by the location of the stimulus presentation the findings showed the anticipated pattern of responses across all groups of mice

(data not shown). That is the attentional components of behaviour were found to be enhanced in trials where the stimulus was presented in the central or near centre apertures, relative to trials where stimulus presentation occurred in the peripheral apertures. These results along with the consistent performance pattern of the correct RT, suggest that the subjects adopted, as anticipated, a scanning and response strategy by using the central position as a point of reference. No difference was found in the pattern of responses between individual WT lines (see Appendix 3.4).

5.3.4 5-CSRTT baseline manipulations

5.3.4.1 Effects of altering ITI length

Interpolating variable ITI durations had a considerable effect on the inhibitory response control of subjects (Figure 5.6). The discriminative response accuracy was not affected by the altering ITI lengths (Figure 5.6A, decreased-ITI session (within-subject test): $F_{2,163}= 1.00$, n.s.; increased-ITI session (within-subject test): $F_{3,207}= 1.56$, n.s.), although a heightened accuracy was found among mice of both *Igf2*-KO lines (relative to WT controls) during the increased-ITI session (between-subject test; $F_{2,69}= 7.17$, $p<0.001$, post hoc tests; *Igf2*-P0 KO: $F_{1,57}= 9.77$, $p<0.001$; *Igf2*-Null KO: $F_{1,48}= 9.02$, $p<0.001$). There were no genotype differences in the amount of omitted trials (Figure 5.6B) when the ITI durations were reduced (between-subject test; $F_{2,69}= 1.57$, n.s.) or increased (between-subject test; $F_{2,69}= 0.39$, n.s), but there was an increase in rates of omissions by all groups as the ITI was lengthened (within-subject test; $F_{3,207}= 5.69$, $p<0.001$). A main effect of genotype was reported for both ITI sessions in the number of premature nose-pokes (Figure 5.6C, between-subject tests; decreased-ITI session: $F_{2,69}= 3.71$, $p<0.05$; increased-ITI sessio: $F_{2,69}= 5.59$, $p<0.01$), indicating a greater response inhibition among *Igf2*-P0 KO mice relative to the WT controls. This effect was against a general background of reduction in premature nose-pokes in the decreased-ITI session (within-subject test; $F_{2,125}= 16.73$, $p<0.001$) and an increase in the increased-ITI session (within-subject test; $F_{3,186}= 22.23$, $p<0.001$).

The number of trials initiated did not differ during sessions where the ITI was altered (within-subject tests; decreased-ITI: $F_{3,207}= 2.36$, n.s.; increased-ITI: $F_{3,207}= 0.66$, n.s.), nor was there any difference found between genotype groups (between-subject test; decreased-ITI: $F_{2,69}= 1.34$, n.s.; increased-ITI: $F_{2,69}= 0.37$, n.s.) and correct RT was not affected by altering ITI lengths (within-subject tests; decreased-

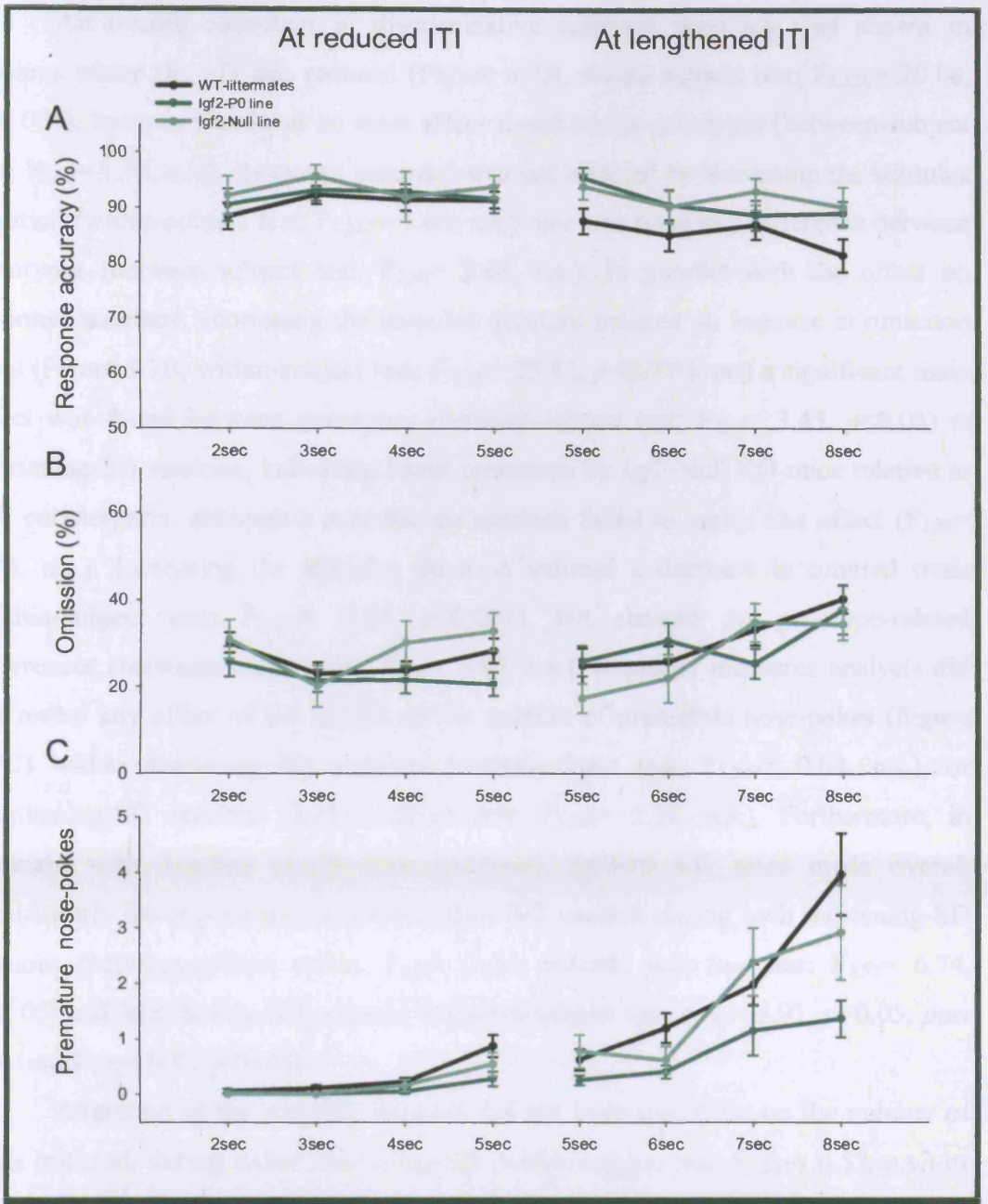

Figure 5.6: *The effects of altering the inter-trial interval (ITI), on 5-CSRTT performance. Mice were presented with sessions where ITI was randomly varied between trials. The figure shows performance in terms of response accuracy* ***(A)****, omitted trials* ***(B)*** *and the number of premature nose-pokes* ***(C)****, at each ITI duration. Data are presented as mean values±SEM.*

ITI session: $F_{3,186}$= 0.68, n.s.; increased-ITI session: $F_{2,172}$= 1.79, n.s.). An analysis conducted on behavioural performance between the separate WT-control lines, did not reveal any significant difference (see Appendix 3.5).

5.3.4.2 Effect of altering SD length

An overall reduction in discriminative response accuracy was shown in sessions where the SD was reduced (Figure 5.7A, within-subject test; $F_{2,120}$= 20.08, p<0.001), however there was no main effect found across genotypes (between-subject test; $F_{2,69}$= 1.97, n.s.). Response accuracy was not affected by increasing the stimulus duration (within-subject test; $F_{3,207}$= 1.03, n.s.), nor was there any difference between genotypes (between-subject test; $F_{2,69}$= 2.03, n.s.). In parallel with the effect on response accuracy, shortening the stimulus duration induced an increase in omission rates (Figure 5.7B, within-subject test; $F_{3,207}$= 25.92, p<0.001), and a significant main effect was found between genotypes (between-subject test; $F_{2,69}$= 3.43, p<0.05) at shortening-SD sessions, indicating fewer omissions by *Igf2*-Null KO mice relative to WT counterparts, although a *post hoc* comparison failed to verify this effect ($F_{1,48}$= 3.76, n.s.). Increasing the stimulus duration induced a decrease in omitted trials (within-subject test; $F_{3,207}$= 6.57, p<0.001), but showed no genotype-related differences (between-subject test; $F_{2,69}$= 0.07, n.s.). Repeated measures analysis did not reveal any effect of SD length on the number of premature nose-pokes (Figure 5.7C) within shortening-SD sessions (within-subject test; $F_{3,207}$= 0.64, n.s.), or lengthening-SD sessions (within-subject test; $F_{3,207}$= 2.58, n.s.). Furthermore, in common with baseline results (see previous), *Igf2*-P0 KO mice made overall significantly fewer premature responses than WT controls during both shortening-SD sessions (between-subject effect; $F_{2,69}$= 3.54, p<0.05; *post hoc* test: $F_{1,57}$= 6.74, p<0.05) and lengthening-SD sessions (between-subject test; $F_{2,69}$= 3.91, p<0.05; *post hoc* test: $F_{1,57}$= 6.57, p<0.05).

Alteration of the stimulus duration did not have any effect on the number of trials initiated, during either shortening-SD (within-subject test; $F_{3,207}$= 0.57, n.s.) or lengthening-SD sessions (within-subject test; $F_{2,130}$= 1.07, n.s.) and there were no differences between genotypes (between-subject tests; shortening-SD: $F_{2,69}$= 0.15, n.s, lengthening-SD: $F_{2,69}$= 2.37, n.s). Correct reaction latencies were also not affected by altering SD during shortening-SD sessions (within-subject test; $F_{2,157}$= 0.18, n.s.) or lengthening-SD sessions (within-subject test; $F_{3,207}$= 2.25, n.s.), and there were no differences noted between genotypes on correct RT in either of the SD-altering sessions (between-subject tests; shortening-SD: $F_{2,69}$= 5.82, n.s.; lengthening-SD:

$F_{2,69}$= 0.56, n.s.). No difference was found between WT-P0 mice and WT-Null mice for any measures, as shown in Appendix 3.6.

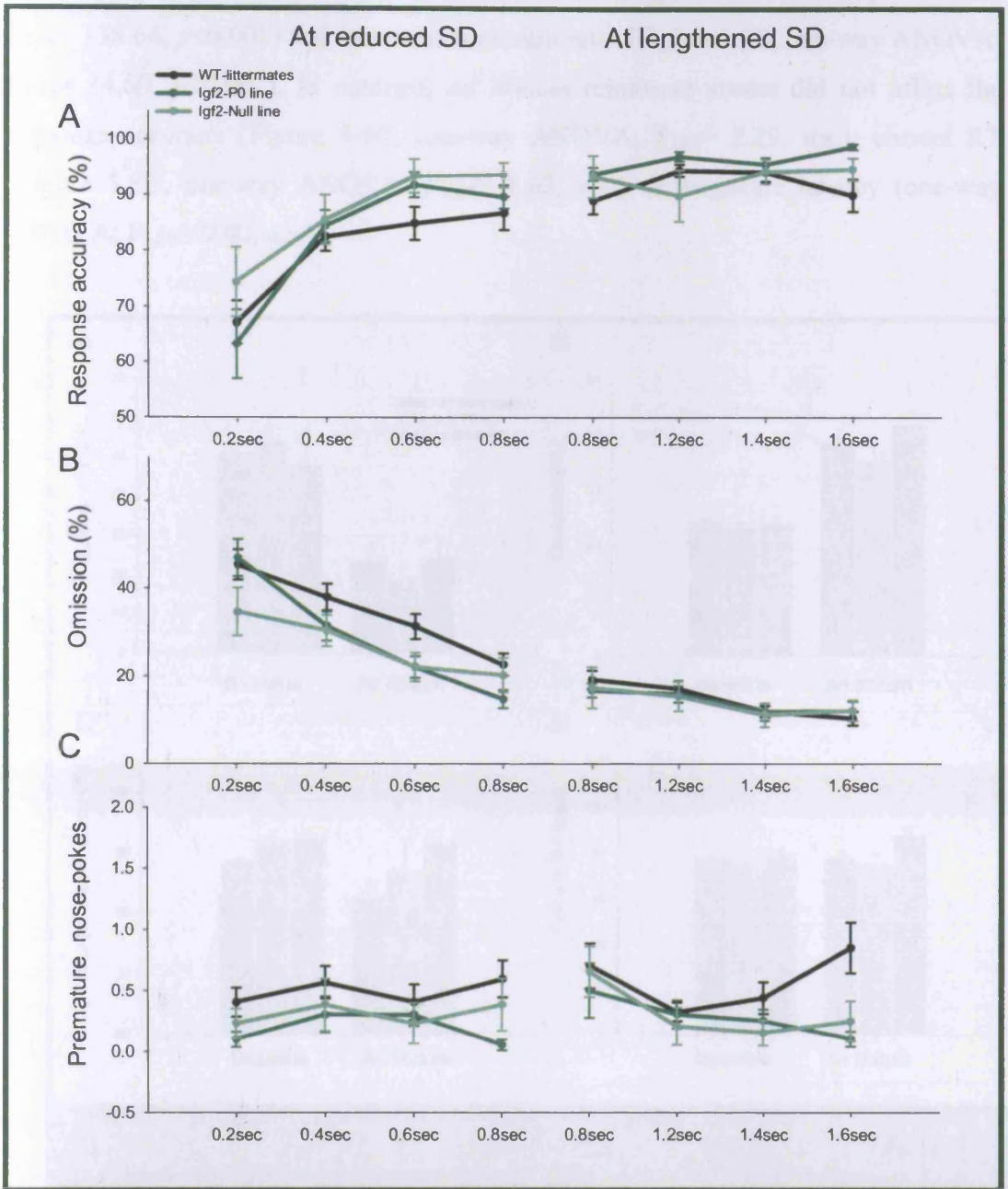

Figure 5.7: *The effects of altering the stimulus duration (SD) on 5-CSRTT performance. Mice were presented with sessions where SD was randomly varied between trials. The figure shows performance in terms of response accuracy* ***(A)****, omitted trials* ***(B)*** *and the number of premature nose-pokes* ***(C)*** *at each SD level. Data are presented as mean values±SEM..*

5.3.4.3 Effects of ad libitum reinforcer access prior to 5-CSRTT testing

Ad libitum access to the condensed milk reinforcer 20 minutes prior to testing significantly reduced the number of trials initiated (Figure 5.8A, one-way ANOVA; $F_{1,69}$= 138.64, p<0.001) and increased omission rates (Figure 5.8B, one-way ANOVA; $F_{1,69}$= 34.69, p<0.001). In contrast, *ad libitum* reinforcer access did not affect the response accuracy (Figure 5.8C, one-way ANOVA; $F_{1,69}$= 2.29, n.s.), correct RT (Figure 5.8D, one-way ANOVA; $F_{1,69}$= 1.63, n.s.) or magazine latency (one-way ANOVA; $F_{1,69}$= 0.00, n.s.).

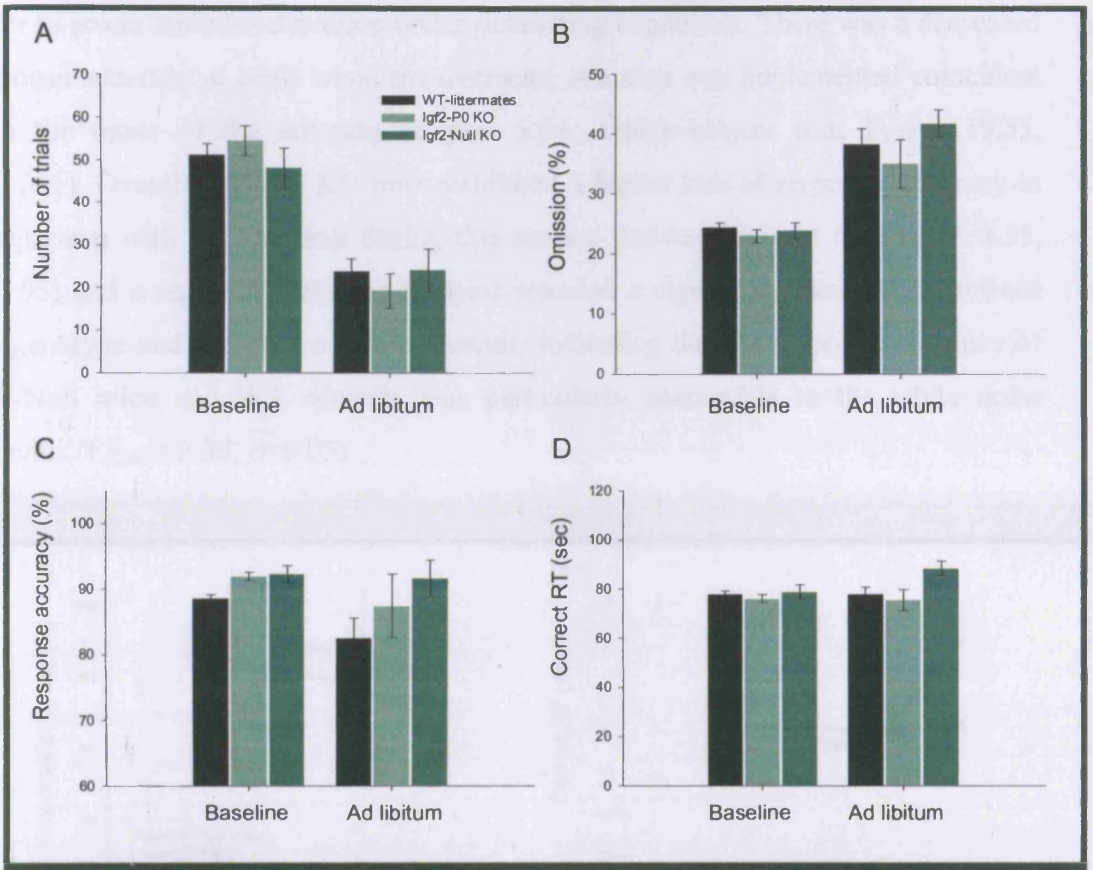

Figure 5.8: *The effects of ad libitum condensed milk access 20 minutes prior to 5-CSRTT testing, on key performance parameters. Graphs show the number of trials initiated* ***(A)****, omitted trials* ***(B)****, response accuracy* ***(C)*** *and correct RT* ***(D)****. Data are presented as means±SEM.*

However, no main effects of genotype were found on these or other behavioural measures (between-subject tests; trials: $F_{2,69}$= 0.05, n.s., omissions: $F_{2,69}$= 0.26, n.s., response accuracy: $F_{2,69}$= 0.85, n.s., correct reaction time: $F_{2,69}$= 2.05, n.s.), the latency to collect the reinforcer was significantly greater among *Igf2*-Null KO

mice (26.58 ± 8.92 sec, Kruskall-Wallis; H_2= 16.45, p<0.001) relative to *Igf2*-P0 KO mice (3.96 ± 2.49 sec, *post hoc* test: U= 33, p<0.001, r=-0.63) and WT controls (4.92 ± 2.29 sec, *post hoc* test: U= 99, p<0.01, r=-0.44). A comparative analysis of 5-CSRTT performance during these sessions between the WT-P0 and WT-Null controls did not reveal any difference (see Appendix 3.7).

5.3.4.4 Effects of 100dB white noise distractor induction

A 100dB white noise distractor stimulus was interpolated at various intervals within the ITI (at the onset of ITI, at 2.5sec into the ITI and at the offset of ITI), in order to assess stimulus detection under distracting conditions. There was a decreased response accuracy at trials when the distractor stimulus was implemented coincident with the onset of the stimulus (Figure 5.9A, within-subject test; $F_{3,181}$= 19.35, p<0.001). Overall, *Igf2*-P0 KO mice exhibited a higher rate of response accuracy in comparison with WT controls during this session (between-subject test; $F_{2,69}$= 3.35, p<0.05) and a repeated measures analysis reported a significant interaction between the genotype and presence of the distractor, indicating that the response accuracy of *Igf2*-Null mice and WT controls was particularly susceptible to the white noise stimulus ($F_{5,181}$= 2.39, p<0.05).

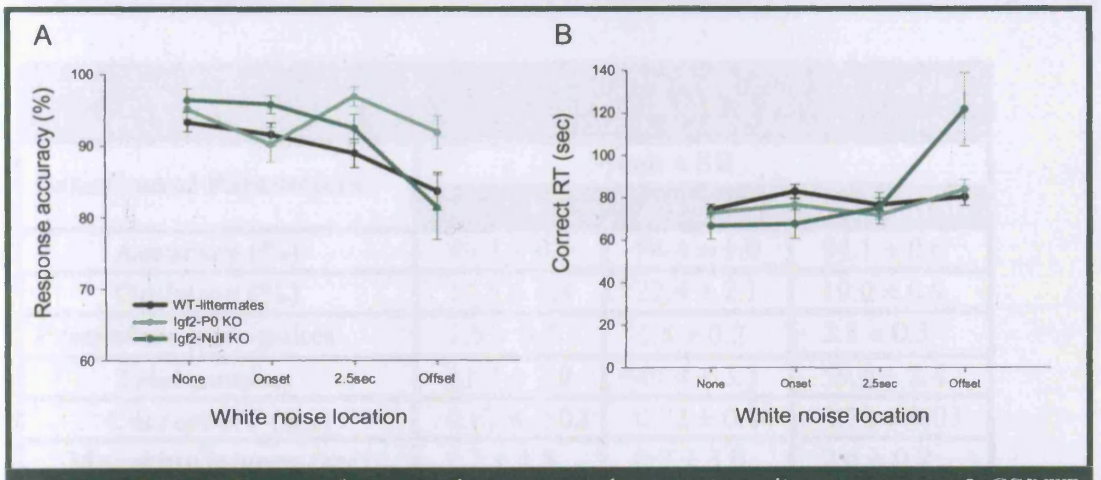

Figure 5.9: *Effects of interpolating a white noise distracter on 5-CSRTT performance. Brief (0.5-sec) 100dB bursts of white noise were presented pseudorandomly on trials at different positions in a trial: at the onset of the ITI, , midway (2.5sec) into the ITI and coinciding with the light stimulus presentation (5sec, offset of the ITI), relative to other trials when no distracter occurred. Graphs show response accuracy* ***(A)*** *and correct RT* ***(B)****. Data are presented as mean values±SEM.*

Furthermore, there was a significant increase in correct RT by *Igf2*-Null KO mice in trials where the white noise stimulus was presented coincident with the stimulus (Figure 5.9B, interaction: $F_{3,98}$= 8.03, p<0.001). In contrast, no difference was found in correct RT when the white noise stimulus was presented earlier during the ITI. Furthermore, the introduction of the white noise stimulus did not have any significant effect in the rate of omissions (within-subject test; $F_{2,136}$= 1.16, n.s.), or in the number of trials initiated (within-subject test; $F_{3,207}$= 0.85, n.s.). In addition, a separate analysis of 5-CSRTT performance during sessions of white noise distraction did not show any difference between the two WT-control lines, as shown in Appendix 3.8.

5.3.5 1-CSRTT baseline performance

Presenting the light stimulus in just the central aperture of the response array, reduced the attentional demand of the task, by excluding the requirement of divided attention. In support of this, the *Igf2*-KO mice, as well as the WT controls, showed an overall improvement in discriminative response accuracy at baseline in the 1-CSRTT relative to 5-CSRTT baseline (within-subject test; $F_{1,69}$= 79.97, p<0.001), although the repeated-measures analysis reported a significant interaction, indicating that the improvement was relatively greater among WT controls ($F_{2,69}$= 6.95, p<0.01). In fact,

Table 5.4: *Behavioural measures of 1-CSRTT performance at baseline level following two consecutive baseline sessions of criterion performance.*

Behavioural Parameters	Mean ± SE		
	Igf2-P0	*Igf2*-Null	WT controls
Accuracy (%)	95.3 ± 0.5	94.4 ± 1.0	94.1 ± 0.6
Omission (%)	17.5 ± 0.8	*22.4 ± 2.1	19.0 ± 0.9
Premature nose-pokes	2.5 ± 0.3	$^{\Psi}$1.4 ± 0.2	2.8 ± 0.3
Trial number	61.7 ± 2.9	*49.4 ± 3.3	59.6 ± 2.4
Correct RT (sec)	0.67 ± 0.02	0.72 ± 0.04	0.70 ± 0.03
Magazine latency (sec)	4.2 ± 1.8	8.7 ± 3.0	2.6 ± 0.7
Duration of eating (sec)	2.7 ± 0.2	2.2 ± 0.5	2.9 ± 0.2
Nose-pokes per trial	1.2 ± 0.1	1.3 ± 0.1	1.6 ± 0.2
Panel pushes per trial	1.8 ± 0.2	1.6 ± 0.1	1.6 ± 0.1

**Significant at p<0.05 to other groups, $^{\Psi}$significant at p<0.05 to Igf2-P0 KO mice only.*

no difference was found in response accuracy across genotypes at baseline sessions during the 1-CSRTT (one-way ANOVA; $F_{2,69}$= 0.95, n.s.), as shown in Table 5.4.

In a similar vein, a significant reduction was observed across all genotypes in the rate of omissions during baseline performance at the 1-CSRTT (within-subject test; $F_{2,69}$= 21.36, p<0.001), relative to 5-CSRTT performance. However, *Igf2*-Null KO mice showed higher rates of omissions at 1-CSRTT baseline session, than *Igf2*-P0 KO mice and WT controls (one-way ANOVA; $F_{2,69}$= 21.36, p<0.001). There was a significant increase in the number of premature nose-pokes emitted (within-subject test; $F_{1,69}$= 4.93, p=<0.05), and in the number of trials initiated (within-subject test; $F_{1,69}$= 5.77, p<0.05), was demonstrated across all genotypes at baseline performance during the 1-CSRTT relative to 5-CSRTT baseline performance, while correct RT was reduced (within-subject test;$F_{1,69}$=20.85, p<0.01). Also of importance, *Igf2*-Null KO mice made fewer premature nose-pokes than *Igf2*-P0 KO mice and WT controls at 1-CSRTT baseline (one-way ANOVA; $F_{2,69}$=4.69, p<0.05), and initiated fewer trials than KO mice of the *Igf2*-P0 line (*post hoc* test: $F_{1,33}$= 6.30, p<0.05). There was no difference however, in correct RT between genotypes ($F_{2,69}$= 0.56, n.s.). One-way ANOVAs (and Kruskall-Wallis test) did not reveal any difference between genotypes on other behavioural parameters; magazine latancy: H_2=5.16, n.s., eating duration: $F_{2,69}$= 0.56, n.s., nose-pokes/trial: $F_{2,69}$= 1.48, n.s., and panel-push/trial: $F_{2,69}$= 0.46, n.s.. Moreover, no difference was found on any of the behavioural parameters between the separate WT lines, see Appendix 5.9.

5.4. Discussion

The present study provides new data into the issue of long-term developmental effects of IUGR due to foetal under-nutrition on cognition and behaviour later in life. The 5-CSRTT was successfully completed by the vast majority of mice, and enabled an extensive analysis of performance along multiple behavioural parameters and across genotypes. Moreover, the introduction of task manipulations affected dissociable aspects of 5-CSRTT performance, in the anticipated manner (see Humby et al., 1999). The key results from this study were that mice of both *Igf2*-KO lines exhibited enhanced response accuracy and impulse control during different stages of 5-CSRTT performance. This effect was more consistent in *Igf2*-P0 KO mice which demonstrated heightened response accuracy during acquisition of the 5-CSRTT, at

baseline, under task conditions where the ITI was increased, and were less distracted by the induction of an auditory stimulus coincident with stimulus presentation. In terms of impulse control, *Igf2*-P0 KO mice were also found to have fewer premature nose-pokes at baseline and during sessions of increased ITI, indictive of enhances behavioural inhibition (i.e. less impulsiveness).

In comparison, mice of the *Igf2*-Null KO line only displayed improved response accuracy relative to WT-controls under conditions where the ITI was increased, but were also found to have reduced rate of omission in sessions in which the SD was shortened. *Igf2*-Null KO mice did not show differences in impulse control but they did demonstrate patterns of behaviour that implied a reduction in levels of motivation, such as fewer trials initiated and greater reaction times during 5-CSRTT acquisition (albeit the difference in reaction time could also be due a locomotor component) and greater latency to collect the reinforcer at baseline. This was particularly true during the simplified 1-CSRTT, where the *Igf2*-Null KO mice initiated a lower number of trials, made fewer premature responses, and showed higher rates of omission at baseline. However, the possible reduction in motivation by *Igf2*-Null KO mice is not necessarily surprising as they have much smaller body sizes and lower body weight (see Chapter III), and thus it is likely that these mice experience satiety earlier than the other mice (cf. drink lower volumes of solution during reinforcer preference test, prior to statistical normalisation). As mentioned in the preceding chapters, the comparison of performance between the *Igf2*-KO lines may provide some information into the distinguishing contributions of foetal nutrient deficiency and growth restriction, to psychiatric disease risk in later life. The fact that subjects of both *Igf2*-KO mouse models demonstrated heightened attentional performance in the present experiment, suggests that the neural substrates underlying this behavioural phenotype are either more susceptible to nutritional insults (relative to the behavioural phenotypes observed solely in *Igf2*-P0 KO mice) or the result of IUGR. By contrast, the enhanced impulse control among Igf2-P0 KO mice might be implicated particularly with foetal nutritional deficiency, and perhaps neurobiological linked with the heightened stress/anxiety-related phenotypes reported in chapters III and IV. This will be discussed further in the General Discussion (Chapter VIII).

As highlighted in the introduction of this chapter, a number of epidemiological studies have previously reported a relationship between IUGR and the risk of ADHD (Schlotz, Jones, Godfrey and Philips, 2008; Lahti et al., 2006; Linnet, 2006; Hawdon,

Hey, Kolvin, and Fundutis, 1990), while other studies of humans have indicated association of nutritional deficiency in early life and ADHD symptoms (Arnold and DiSilvestro, 2005; Arnold et al., 2005; Bilici et al., 2004; Brophy, 1986). Attentional problems are one of the main symptoms of ADHD, and people with ADHD have been shown to have difficulties both in the ability to maintain attention to stimuli that are widely spaced in time (i.e. sustained attention) (Johansen, Aase, Meyer and Sagvolden, 2002), as well as in the ability to maintain attention on a stimulus in the face of other (irrelevant) distracting stimuli (i.e. selective attention) (Gomarus, Wijers, Minderaa and Althous, 2009). In the present study however, *Igf2*-P0 KO mice displayed evidence of improvements in aspects of attentional function during the 5-CSRTT; this was not the case during the simplified 1-CSRTT. The differential results obtained between the two versions of this task, might infer that this improvement of attentional function is confined to aspects of selective attention (i.e. attention over space), rather than sustained attention (i.e. attention over time). Conversely, the more circumstantial improvement in accuracy, noted during both 5-CSRTT and 1-CSRTT in *Igf2*-Null KO mice, could possibly be related to more general improvement of attentional aspects. Although a heightened motivational level could in general contribute to improvements to some of the aspects of performance on the 5-CSRTT, such as higher response accuracy, a greater number of trials initiated or a reduction in omission rate, it highly unlikely that this is the case for the *Igf2*-KO mice. The *Igf2*-P0 KO mice did not display differences for any of the motivational-related indices. Firstly, there was no difference observed in the volume of reinforcer consumed during the reinforcer habituation between the *Igf2*-P0 KO mice and WT controls, and secondly, the number of trials initiated throughout the 5-CSRTT did not vary between the *Igf2*-P0 KO mice and WT-controls, and the latencies of stimulus response and collection of reinforcer were also equivalent. Moreover, the *Igf2*-Null KO mice displayed, in fact, evidence of less motivational level during the both the 5-CSRTT and the 1-CSRTT, as indicated by fewer number of trials intitiated.

Even though impulsive behaviour is another one of the hallmark traits of ADHD, impulsivity is generally conceived to comprise a variety of different (but related) cognitive/behavioural properties, rather than being a unitary construct (Winstanley et al., 2006). A variety of behavioural paradigms have been devised in effort to assay these different aspects of impulsivity. While the 5-CSRTT provides a measurement of a certain aspect of response inhibition that entails a cognitive

component (i.e. impulse control) as the subject is required to make a decision of response, another behavioural paradigm known as the Stop signal serial reaction time task (SSRTT) indices an aspect of response inhibition that relies heavily on motor components (response control) since in this task the subject is required to abort an ongoing prepotent response (Logan and Cowan, 1984). One of the concerns which the findings of present study raise, is whether the reduction of premature nose-pokes observed among *Igf2*-P0 KO mice solely reflects a confined improvement of impulse control, or if it represents a greater behavioural inhibition on a broader scale that might involve improved response control in addition. This issue will be addressed in the next chapter (Chapter VI), by testing the *Igf2*-KO mice along with their WT-littermate controls on the SSRTT.

The discrepancy observed between the results of the current study and findings from the literature on ADHD, especially in terms of IUGR, is not necessarily paradoxical, but rather highlights the importance to draw a twofold distinction in this line of research. Firstly, brain development in rodents, as well as humans, progresses in region-specific manner, thus different brain regions undergo critical periods of development at varying time points (e.g., caudal parts of the brain develop generally earlier than rostral parts, and subcortical structures mature earlier than cortical). Therefore, it is pivotal that researchers do not premise that a certain environmental stimulus occurring in foetal life will give rise to the same or even similar central nervous system profile, in terms of neurobiology, cognition or behaviour, as that environmental insult would when acting in early postnatal life. Secondly, instead of merely examining the association between IUGR and adult mental health as much of the previous research has focused on, it is imperative the future research concentrates on how the different causal agents of IUGR impact neurodevelopment, cognition and behaviour in later life.

In recent years, a number of lesion studies have managed to unveil some of the neural mechanisms that are critically involved in 5-CSRTT performance. The link between lesions to the medial parts of the prefrontal cortex and striatum on one hand, and significant deficits in response accuracy on the other (Muir, Robbins and Everitt, 1996; Rogers, Baunez, Everitt and Robbins, 2001), implicates the importance of these cerebral regions in modulating attentional functions. There is also evidence indicating that the medial striatum might play a key role in response inhibition, as reflected by a heightened number of premature nose-pokes following lesions to this region (Rogers

et al., 2001). Further to this, studies using neuropharmacological manipulations, have implicated many of the main neurotransmitter systems in 5-CSRTT performance in rodents. In fact, cholinergic, catecholaminergic (i.e. dopamine and noradrenaline) and serotonergic systems have all been attributed a role in response accuracy on the 5-CSRTT (see Robbins, 2002). For instance, agonists of excitatory cholinergic and catecholaminergic receptor subtypes have been shown to improve choice accuracy (Muir, Robbins and Everitt, 1995; Puumala, Riekkinen and Sirvio, 1997; Granon et al, 2000), whereas administrations of excitotoxins or antagonist of these neurotransmitter receptors have produced in deficits on attentional performance (Muir, Robbins and Everitt, 1994; Jones, Barnes, Kirkby and Higgins, 1995; Puumala et al., 1997; Granon et al., 2000). On the other hand, the involvement of the 5-HT system in 5-CSRTT performance has primarily been investigated in relation to impulse control; despite some evidence of a role for 5-HT in response accuracy also (Harrison, Everitt and Robbins, 1997a; Jakala et al., 1992). Although some of the research for the regulatory role of 5-HT in impulsivity has produced contradicting findings, depletion of 5-HT in the forebrain has been demonstrated to increase premature responses on the 5-CSRTT (Harrison, Everitt and Robbins, 1997b). Similar effects were noticed in the 5-CSRTT, following administration of 8-OH-DAT, an agonist of the inhibitory 5-HT_{1A} receptor (Carli and Samanin, 2000) or administration of SB242084, a 5-HT_{2C} specific antagonist, but an administration of a 5-HT_{2A} specific antagonist resulted in the opposite (Winstanley, Theobald, Dalley, Glennon and Robbins, 2004).

In light of the above findings, it is conceivable that the malnutrition-induced IUGR, could possibly have re-programmed the developmental trajectories of the prefrontal cortex and/or the striatum (as well as other brain regions), affecting one or possibly more of these neurostransmitter pathways. Such developmental alterations, might in turn, have entailed lasting morphological and neurophsysiological changes at these cerebral loci, which underpin the enhanced attentional functions and impulse control observed in mice of the *Igf2*-P0 KO line. It is of paramount importance however that further research is carried out to investigate differences in cerebral morphology and/or functions of neurotransmitter pathways, between the *Igf2*-P0 KO mice (and possibly the *Igf2*-Null KO mice) and their WT-littermate controls. This research effort could encompass; histological examination of region-specific neuronal integrity, measurements of regional neurotransmitter levels, as well as confined exploration of gene expression. The outcome of such research could provide

important insight into how brain development is compromised by placental deficiency in intrauterine life, as well as give rise to a greater clarity of the behavioural profile exhibited by the *Igf2*-P0 KO mice. This issue will be addressed in Chapter VIII.

5.4.1 Summary of key results from Chapter V

- *Igf2*-P0 KO mice displayed a significantly improved discriminative response accuracy in during both the acquisition of the 5-CSRTT and at baseline, relative to WT controls.
- *Igf2*-P0 KO mice showed also better response accuracy than WT controls under task conditions where the ITI was increased and were less distracted by the induction of an auditory stimulus coincident with stimulus presentation.
- *Igf2*-P0 KO mice made fewer premature nose-pokes than WT controls in the 5-CSRTT, at baseline and during sessions of increased ITI.
- *Igf2*-Null KO mice showed improved response accuracy relative to WT controls under condition where the ITI was increased, during both the 5-CSRTT and the simplified 1-CSRTT.
- There was a reduction in rate of omission among *Igf2*-Null KO mice in sessions in which the SD was shortened, and response accuracy was heightened under conditions of white noise distraction during the 1-CSRTT (data not shown).
- *Igf2*-Null KO mice initiated fewer trials and showed greater reaction time during the acquisition of the 5-CSRTT, and greater latency to collect the reinforcer at baseline.
- During the 1-CSRTT, *Igf2*-Null KO mice intitiated fewer number of trials, made fewer premature nose-pokes, and showed higher rates of omissions at baseline.

Chapter VI

Further Investigation of Behavioural Inhibition in Igf2-P0 and Igf2-Null Knockout Mice: The Stop-Signal Reaction Time Task

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6.1 Introduction

One of the main results in the 5-CSRTT, discussed in the previous chapter, was an enhanced behavioural inhibition in mice of the growth restricted *Igf2*-P0 KO line relative to *Igf2*-Null KO mice and wild-type (WT) controls. These results were unexpected, with regard to previous epidemiological studies which have consistently reported inverse correlations between intrauterine growth retardation (IUGR) and measures of impulsivity in childhood, and similarly the risk of attention deficit/hyperactivity disorder (ADHD) (Breslau, 1995; Hack et al., 1992; Pharoah, Stevenson, Cooke and Stevenson, 1994; Wichers et al., 2002). Therefore, the purpose of the current chapter was to further investigate the response control of *Igf2*-KO mice relative to their WT littermates to shed light on the scope of the improved behavioural inhibition, previously noted among the *Igf2*-P0 KO mice.

Impulsivity has been highlighted as one of the most fundamental clinical symptoms of ADHD, along with inattention and hyperactivity (DSM-IV-TR, 2000). Impulsivity refers to lack of behavioural inhibition, including actions that are poorly conceived, premature, unnecessarily risky or contextually inappropriate and frequently result in an unfavourable outcome (Evenden, 1999a). Current theoretical frameworks of behavioural inhibition propose that a lack of impulse control arises as the consequence of an underactive inhibitory system which fails to provide transient suppression of conditioned responses and reflexes that would enable slower cognitive processes to regulate behaviour (Winstanley, Eagle and Robbins, 2006). However, on the basis of the diversity of behaviour which impulsivity encompasses there is good reason to consider it to be a heterogeneous aspect of behaviour possibly involving several different neurobiological substrates rather than a unitary construct (Evenden, 1999b). Nevertheless, by focusing on certain circumscribed behavioural aspects of impulsivity, a variety of behavioural paradigms have been successfully established, in

order to measure impulsivity in both human and non-humans subjects (Winstanley et al., 2006).

Laboratory measurements of impulsivity have been primarily based on two animal models (Swann, Bjork, Moeller and Dougherty, 2002). The first model measures impulsive choice, in terms of the inability of subjects to delay a reward, leading to an increased tendency to choose immediate small rewards over larger delayed one (Monterosso and Ainslie, 1999). The second model on the other hand, examines the inability to inhibit a prepotent response, leading to errors of commissions (Evenden, 1999c). One of the tasks that make use of the latter measurement to study response control is the Stop signal reaction time task (SSRTT, Logan, Cowan and Davis, 1984), where the subject is required to perform speeded respond when cued to do so by a "Go" signal (Go-trials). However, on a subset of trials the subject is required to inhibit responding when the "Go" signal is briefly followed by the presentation of a "Stop" signal (Stop-trials). By outnumbering the Stop-trials considerably with Go-trials, responding rather than inhibiting is made pre-potent. The closer the Stop signal is presented in time relative to the moment of responding (i.e. "goings"), the more difficult it is for the subject to inhibit their behaviour. Many of the current theoretical models of ADHD have highlighted the importance of the ability of individuals to stop an ongoing response (Alderson, Rapport and Kofler, 2007), and research using the SSRTT has repeatedly revealed that children and adults with ADHD tend to have longer Stop signal reaction times relative to normal controls (see Winstanley et al, 2006 and Lijffijt, Kenemans, Verbaten and van-Engeland, 2005 for review). Therefore, the Stop signal paradigm has been widely adopted for ADHD research, due to it exceptional ability to assay theoretically important cognitive processes by means of the Stop signal reaction time metric.

As mentioned, *Igf2*-P0 KO mice showed reductions in premature nose-pokes at baseline conditions and during manipulation of inter-trial interval length in the 5-CSRTT suggesting improved behavioural inhibition. Although both the 5-CSRTT and the SSRTT provide quantifiable indices of behavioural inhibition, these behavioural tasks assay different aspects of inhibitory (or impulsive) behaviour (see Nigg, 2000 for discussion). While the 5-CSRTT task assesses impulse control, i.e. the ability of a subject to inhibit a pre-potent response in anticipation of food reward, the SSRTT examines response control or the ability of subjects to inhibit an ongoing pre-potent

response. Comparisons in measures of response control between the two tasks in rodents is not straightforward as few studies have been reported for the SSRTT to date, whereas 5-CSRTT performance has been more extensively studied (see Robbins, 2002). In terms of the major forebrain neurotransmitter systems, evidence from the SSRTT has demonstrated improvements in impulse control after the administration of methylphenidate and atomoxetine, reuptake inhibitors for dopamine and noradrenaline, respectively (Eagle, Tufft, Goodchild and Robbins, 2007; Turner, Clark, Dowson, Robbins and Sahakian, 2004; Chamberlain et al., 2009; Robinson et al., 2008, respectively), whereas selective 5-HT reuptake inhibitors show little behavioural effects (Chamberlain et al., 2006; Clark et al., 2005; Eagle et al., 2009). This is in contrast to the 5-CSRTT, where manipulations of the 5-HT system have profound effects on the main measures of impulse control (see Robbins, 2002). Where the same pharmacological agent (D-amphetamine) has been used in both tasks, differential effects have been found whereby impulsivity is increased in the 5-CSRTT (Robbins, 2002) but no effect on impulse control is found in the SSRTT (Eagle and Robbins, 2003). Thus the differences in the nature of response control are also reflected by differential effects of pharmacological manipulations, suggesting that different brain mechanisms/systems may be involved in the control of responding in each task. If the *Igf2*-P0 KO mice do not show any evidence of improvement response control during the SSRTT, it could be hypothesized that the enhanced behavioural inhibition (as observed during the 5-CSRTT) is somewhat restricted to improved cognitive control. On the other hand, improved response control in the SSRTT could signify that *Igf2*-P0 KO mice show a broader impulsivity-related phenotype.

6.2. Materials and Methods

Full methodological descriptions can be found in the relevant sub-sections of the General Methods Chapter (Chapter II).

6.2.1 Subjects and animal husbandry

The SSRTT task was carried out on a total number of 75 male mice (aged 16-18 months at the onset of experiment) generated in the Behavioural Neuroscience Laboratory at Cardiff University (see table 6.1). The subjects were derived from a

crossbreeding of background strain of C57BL/6*129 (carrying either the mutant *Igf2*-P0 allele or the *Igf2*-Null allele) with CD1 females, as previously described in Chapter II.

Table 6.1: *The sample size and genotype of the total litter cohort which began and completed the SSRTT.*

Status	Genotype and sample size (*n*)		
	Igf2-P0	*Igf2*-Null	WT (WT-P0/WT-Null)
Number of subjects to begin the SSRTT	29	16	41 (20/21)
Number of subjects to complete the SSRTT	16	8	30 (12/18)

The mice were housed in littermates groups of two to five animals per cage, under temperature- and humidity-controlled conditions, with a 12-hour light: 12-hour dark cycle (lights on at 07:30 hours). Mice were 8 weeks old at the onset of the experiment, and had access to standard laboratory chow and water *ad libitum*, but restricted to 2-hours access per day when behavioural experiments commenced, as described previously 2.4.4. The body weight of each mouse was monitored regularly for indication of health status (see Chapter II, section 2.4.2). All experimental procedures were conducted under licenses issued by the Home Office (U.K.) in compliance with the Animals (Scientific Procedures) Act 1986 and local ethics committee.

6.2.2 Behavioural methods

6.2.2.1 Habituation to the reinforcer

Once body weight had stabilised under the water restriction schedule, the habituation/preference testing of the condensed milk reinforcer was established. Testing was carried out as previously described (Chapter II, section 2.4.5), with the main index of preference being the amount of reinforcer (in terms of percentage of total fluid consumption) consumed on the final day of testing.

6.2.2.2 The Stop signal reaction time task

In the SSRT task, an occasional presentation of a Stop signal indicates a pending 'Go' response to be cancelled and provides an estimation of the time needed to withdraw the response (see Figure 6.1). The Stop signal reaction time (SSRT) was determined by measurable parameters: the distribution of the Go-trial reaction time (Go-RT) and the accuracy of stopping during Stop trials. The testing took place in nine-aperture operant chambers previously modified for use in mice (Humby et al., 1999), with seven of the nine apertures in the response array covered. The mice performed one 20-minute session per day with a maximum of 100 trials per session.

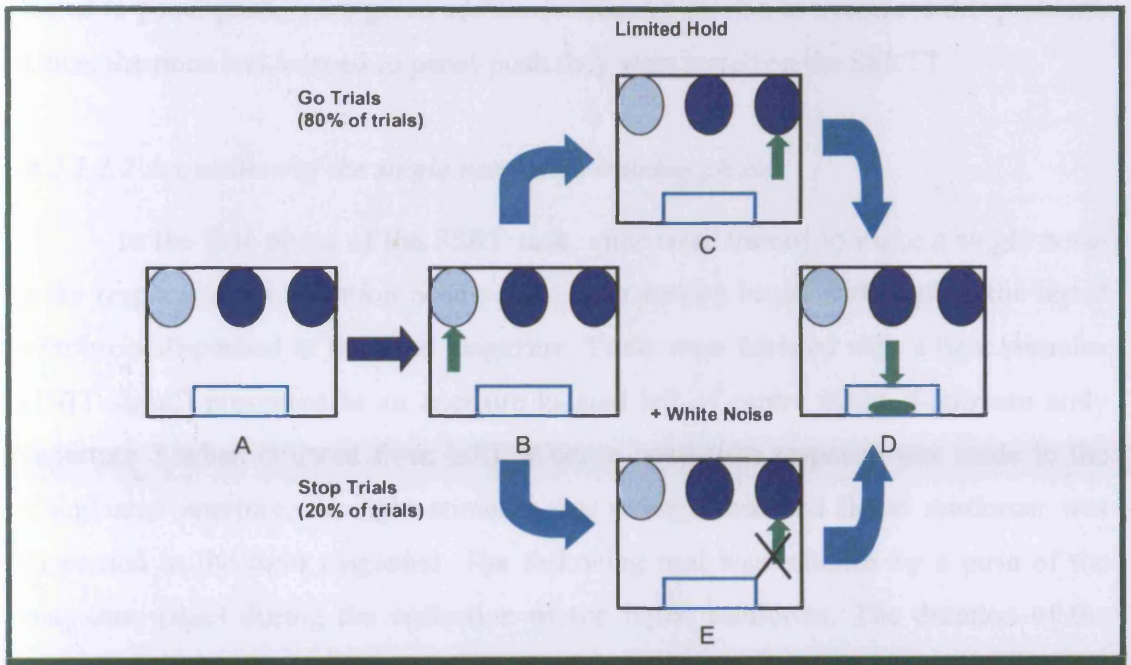

Figure 6.1: *The SSRTT procedure. A stimulus light presented in an aperture on the left side of the response array signals the beginning of the trial* ***(A)****. A nose-poke in the illuminated aperture on Go-trials* ***(B)*** *initiates the a stimulus light presented in an aperture on the right side of the response array* ***(C)****. A nose-poke in the right aperture results in the delivery of the liquid reinforcer* ***(D)****. On Stop-trials, a burst of white noise is presented following a nose-poke in the left aperture which requires the subject to refrain from responding to the right stimulus light* ***(E)****. Incorrect responses (failure to nose-poke in both apertures on Go-trials or a nose-poke in right aperture on stop trials) results in a time-out period.*

6.2.2.2.1 Initial behavioural training

The mice were subjected to training sessions on a daily basis that were scheduled at the same time of the day and with each mouse consistently allocated to

the same operant chamber. Shaping was carried out over five consecutive sessions. In the first three sessions, mice were habituated to the operant chamber and trained to press the magazine panel, in order to get an access to the liquid reinforcer. In these sessions, 40μl of the liquid reinforcer was delivered into the food magazine every 30 seconds throughout the session, indicated by the illumination of the tray light coincident to the food delivery. The panel of the food magazine was wedged open, allowing easy access to the reinforcer, while the apertures in the response array were blocked with a transparent plastic film. During the final two sessions of shaping, the panel of the food magazine was not wedged open, constraining the mice into learning to push the panel (i.e. 'panel-push') in order to gain access to the reinforcer. Mice that failed to panel-push, were given additional training session to overcome this problem. Once, the mice had learned to panel-push they were tested on the SSRTT.

6.2.2.2.2 Acquisition of the single nose-poke training phase

In the first phase of the SSRT task, mice were trained to make a single nose-poke response; 'the initiation nose-poke'. Each session began with 40μl of the liquid reinforcer dispensed in the food magazine. Trials were initiated with a light stimulus (INIT-signal) presented in an aperture located left of centre in the 9-aperture array (aperture 3 when counted from left). When a nose-poke response was made in the illuminated aperture, the light stimulus was extinguished and liquid reinforcer was dispensed in the food magazine. The following trial was initiated by a push of the magazine panel during the collection of the liquid reinforcer. The duration of the initiation stimulus was initially set at 30 seconds but was automatically reduced to 10 seconds in 5-sec decrements in accordance to the performance of the mice (on five consecutive correct trials). If the mice failed to make nose-poke response during the stimulus presentation, the mice received a time-out period of 5 seconds, and the trials was recorded as non-started. On reaching a stable performance with an initiation stimulus duration of 10 seconds and completion of <70% of trials (out of 100 trials), the mice were moved to the *double nose-poke* stage.

6.2.2.2.3 Acquisition of double nose-poke training phase (Go trials)

Mice were trained to perform a rapid reaction time response (e.g. double nose-poke) from the left initiation aperture to the right 'Go' aperture, termed a "Go-

response". The session began with the delivery of the liquid reinforcer. A light stimulus was presented immediately in the initiation aperture on the left side of the response array and in this phase, the initiation stimulus was fixed at 10 seconds. When the mice made a nose-poke response in the illuminated aperture, the light stimulus was extinguished and a second light stimulus was presented in an aperture located on the right side of the response array (aperture 6 when counted from left). If another nose-poke response was then made to illuminated aperture on the right side, the light stimulus extinguished and the liquid reinforcer was delivered. The limited hold of the second light stimulus (Go-LH) was initially set at 60 seconds and decreased to a Go-LH duration equivalent to the mean Go reaction time (mean Go-RT, *m*Go-RT) for each subject with the addition of 0.3 second, allowing individual differences to be taken into account. Decrements in Go-LH were in 5-sec intervals, following five consecutive trials. Once the mice had performed two sessions at their own individually fixed Go-LH duration, while completing >70% of the trials, they were moved on to the next stage.

6.2.2.2.4 Introduction of the Stop signal trials

At this phase of the experiment, Stop signals were presented pseudo-randomly on 20% of the trials during each session, thus the Stop signal delay (SSD) was zero (0%). The Stop signal was presented as a burst of white noise (65dB for 0.3 second), at a time coincident with completion of the initiation nose-poke response (which indicated that the subjects had to withhold their second nose-poke response (i.e. stopping) in order for the liquid reinforcer to be delivered). The time duration for which the mice had to withhold their response (Stop-LH), was initially set at 0.35 second, a period slightly longer than the duration of the white noise burst, thus enabling the mice to hear the delivery of the liquid reinforcer. The Stop-LH was then increased as performance improved in 0.1-sec intervals for every five consecutive correct trials to a value ranging between 0.7-sec to 1.1-sec, depending on the performance of each individual subjects. In order to reach the baseline performance criteria, subjects needed to; initiate more than 70% of the trials, correctly stopping (withholding response) at least 65% of the time with a minimum Stop-LH of 0.7 second, have greater than 70% accuracy in Go-responses, and less than 50% omission.

6.2.2.2.4 The signal delay phase

Once a stable baseline performance was reached following the initial training, the response control capability of the mice was investigated by varying the SSD. Each experimental set was implemented following a single baseline session with criteria performance, and the introduction of the SSD occurred at several different temporal locations that varied between sessions. In baseline sessions the Stop signal was presented at a time coincident with the initiation nose-poke (i.e. 0% with respect to the *m*Go-Rt for each subject, thus SSD=0%). The present SSRTT study utilized a programme that provided an on-line monitoring of the Go-response reaction time (Go-RT) for each mouse and calculated the SSD as a percentage of the on-going Go-RT for each individual subject. As the individual session progressed onwards, the mean average Go-RT for every subject was based on an increasing number of Go-RT from the previous Go-trials of that same session. The SSDs were presented in the following order; SSD=70% of *m*Go-RT (i.e. mean Go RT*0.7), SSD=10% of *m*Go-RT, SSD=90% of *m*Go-RT, SSD=30% of *m*Go-RT, SSD=50% of *m*Go-RT, SSD=80% of *m*Go-RT, SSD=60% of *m*Go-RT, SSD=20% of *m*Go-RT and SSD=40% of *m*Go-RT.

6.2.2.3 Calculating the Stop-signal reaction time

SSRT represents a 'virtual' measures of the latency to stop a response and as such cannot be measured directly, thus it was determined by key task parameters (the distribution of the Go-RT and the accuracy of stopping during Stop trials) according to the protocol previously described by Logan (1994), and adapted for experiments in rats (Eagles and Robbins, 2003). Therefore, to calculate the SSRT, Go-RTs were first rank ordered from shortest to longest and the *n*th Go-RT was then found, where *n* was obtained by multiplying the number of RTs in the distribution by the probability of stopping correctly. The *n* value provides an estimate of the time point at which the stopping response finished comparative to the onset of the Go signal. The SSRT estimation, i.e. the time point at which the stopping response finished, relative to the Stop signal, was acquired by subtracting the SSD from the *n* value, where SSD was calculated as the *m*Go-RT for that session * SSD value as a percentage. This was repeated for each subject for each delay, and the resulting mean was obtained for all genotype groups.

6.2.2.4 Behavioural measures

Task acquisition of the SSRTT was determined by the number of sessions required for each subject to reach SSRTT baseline performance, and the number of sessions needed to complete each of SSRTT acquisition phases. A number of different behavioural measures were recorded for each session, in order to establish a relatively broad behavioural profile of the *Igf2*-KO lines. The primary indices of aspects of response control were the percentage of response accuracy at Stop-trials (i.e. correct Stop responses/(correct Stop-responses + incorrect Stop-responses) and the reaction time of correct Stop-responses (SSRT), during the acquisition of Stop signal trials, at baseline performance and sessions of varying SSDs. Response accuracy and reaction time of correct responses during Go-trials (Go reaction time) were also registered from the Go-trial acquisition phase and onwards. While recording response accuracy during Go-trials, provided a measurement of attentional function, it also enabled examination of response pattern among the subjects in order to detect potential response bias (i.e. tendency of a subject to solely make either Stop- or Go-responses). Other measures of interest included: number of trials initiated (reflecting overall performance of subjects), percentage of trials completed (indexing levels of motivation), the number of INIT nose-pokes, Go nose-pokes, total nose-pokes and panel-pushes, per trial.

6.2.3 Statistical analysis

The behavioural data were subjected to ANOVA by either using a one-way design to determine between-subjects effect or general linear design to allow for examination within-group effect. The genotype of subjects was primarily defined as the between-subject factor throughout the study. However, the order of session (i.e. first session vs. last session) within an acquisition phase was defined as the within-subject factor during SSRTT acquisition, while the location of the SSD within the Go-reaction time of subjects was assigned as the within-subject parameter during the SSRTT.

Data from the separate WT lines were clustered together for analysis in order to increase the statistical power and for other reasons previously mentioned in Chapter II, section 2.4.7. However, every analysis of SSRTT performance during task acquisition, baseline and SSD alteration, was conducted separately for the WT-P0 and

WT-Null mice to ensure behavioural homogeneity between the two WT lines. Independent T-tests were used to examine possible genotype difference, whereas a repeated measures analysis was used to test for any interactions. The results of these analyses are presented in Appendix VI, unless otherwise stated.

6.3. Results

6.3.1 Reinforcer habituation and preference testing

The analysis of the milk reinforcer preference and total volume consumption was conducted on mice that completed the SSRTT. The preference for the condensed milk reinforcer increased rapidly across the four habituation sessions (Figure 6.2A, within-subject test; $F_{2,104}= 93.71$, $p<0.001$), but there was no difference observed across genotypes (between-subject test; $F_{2,51}= 0.51$, n.s.). By the final habituation session, mice of all three genotypes had displayed a strong preference for the condensed milk reinforcer (*Igf2*-P0 KO: $M= 92\%$, $SE= 0.65$, $t(15)= 62.99$, $p< 0.001$, $r= 0.996$; *Igf2*-Null KO: $M= 89\%$, $SE= 2.54$, $t(7)= 15.49$, $p< 0.001$, $r= 0.99$; WT controls: $M= 89\%$, $SE= 2.33$, $t(29)= 17.04$, $p< 0.001$, $r= 0.91$) with over 85% preference on average. A one-way ANOVA at day six, did not reveal any difference in reinforcer preference between genotypes ($F_{2,51}= 0.54$, n.s.).

Moreover, a steady rise in the total volume of liquid consumed over the course of the reinforcer habituation was noted among all subjects (Figure 6.2B, within-subject test; $F_{4,194}= 49.76$, $p< 0.001$). In contrast, the total volume of liquid consumed in the final session of the reinforcer habituation, was significantly reduced among mice of the *Igf2*-Null KO line relative to *Igf2*-P0 KO mice and WT-littermate controls (one-way ANOVA; $F_{2,51}= 5.56$, $p<0.01$). However, this difference did not persist following a statistical-correction for body weight (one-way ANOVA; $F_{2,51}= 2.27$, n.s.). Comparison between the WT-littermate controls of the two mutant lines (i.e. WT-P0 littermates and WT-Null littermates), did not reveal any difference in the preference for the milk reinforcer or in the overall volume of liquid consumed (Appendix 4.1).

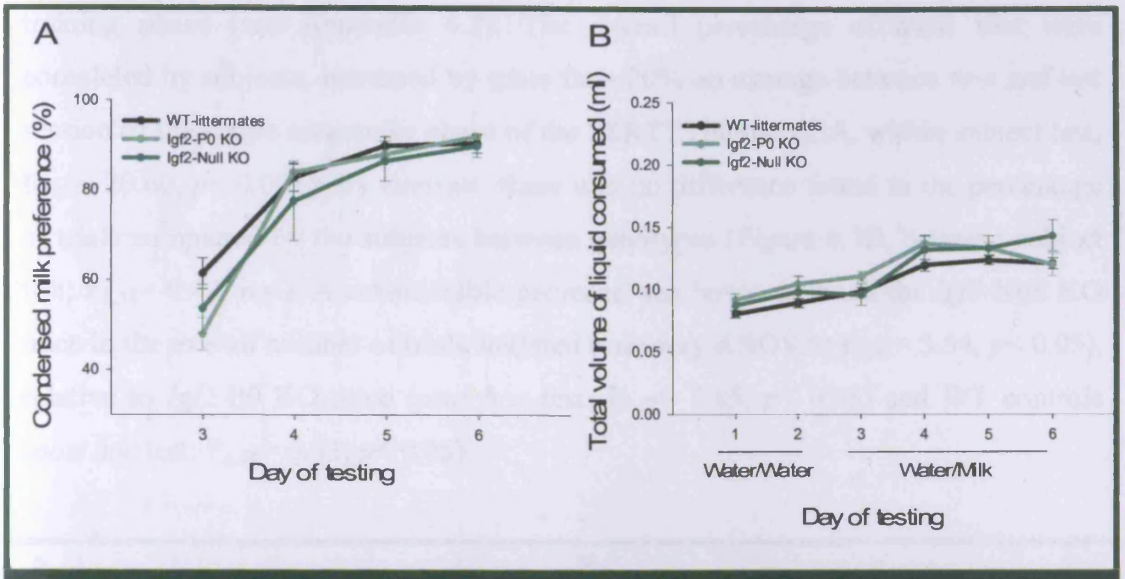

Figure 6.2: *Reinforcer habituation. Subjects were only presented with water during the first two days of reinforcer habituation, but in the following four days an option between water and condensed milk (10%) was present.* ***(A)*** *Subjects established a relatively strong preference for the condensed milk reinforcer by day four, reaching levels >85%.* ***(B)*** *Total liquid consumption (water and condensed milk combined), normalised for body weight of the subjects, gradually increased over the course of the reinforcer habituation sessions. For subject N see methods section 6.2.1. Data are presented as mean±SEM.*

6.3.2 Acquisition of the SSRTT

Of the cohort of 75 subjects that commenced the SSRTT, 54 subjects managed to reach baseline and complete all task manipulations within 125 sessions (see Table 6.1). There was no difference observed between genotype in SSRTT acquisition, in terms of the number of sessions required to reach baseline performance (*Igf2*-P0 KO: 64.56 ± 3.93 day, *Igf2*-Null KO: 59.5 ± 3.0 day, WT controls: 57.00 ± 2.27 day, $F_{2,51}$= 0.58, n.s.).

6.3.2.1 Acquisition of the single nose-poke training phase

In the single nose-poke training phase of the SSRTT, a single nose-poke to an illuminated aperture located on the left side (INIT-signal) was rewarded with a delivery of the reinforcer. All subjects completed the single nose-poke phase of the SSRTT acquisition on average by Session 46, with no significant difference observed between genotype (one-way ANOVA; $F_{2,51}$= 0.08, n.s.). Similarly, no difference was noted between the WT controls of the separate mutant lines in the acquisition of this

training phase (see Appendix 4.2). The overall percentage of trials that were completed by subjects, increased by more than 20% on average between first and last session of the single nose-poke phase of the SSRTT (Figure 6.3A, within-subject test; $F_{1,51}$= 20.60, p< 0.001). By contrast, there was no difference found in the percentage of trials completed by the subjects between genotypes (Figure 6.3B, between-subject test; $F_{2,51}$= 0.49, n.s.). A considerable decrease was however noted for *Igf2*-Null KO mice in the overall number of trials initiated (one-way ANOVA; $F_{2,51}$= 3.54, p< 0.05), relative to *Igf2*-P0 KO mice (*post hoc* test: $F_{1,22}$= 5.85, p< 0.05) and WT controls (*post hoc* test: $F_{1,36}$= 6.33, p< 0.05).

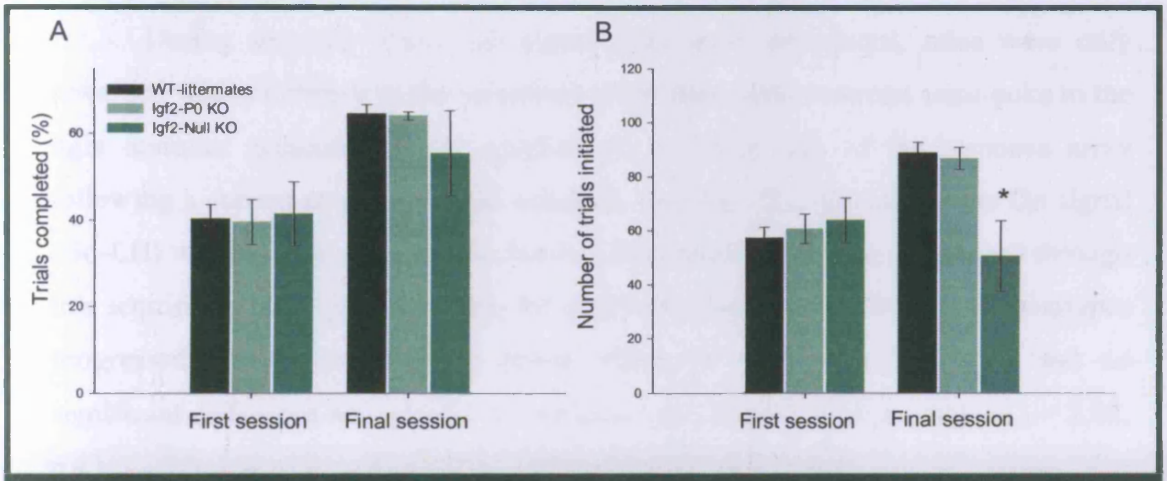

Figure 6.3: *Acquisition of the single nose-poke phase. The performance of subjects during at the first and final sessions of the single nose-poke phase of the SSRTT acquisition, in regards of the percentage of trials completed by the subjects* ***(A)*** *and the total number of trials initiated* ***(B)****. Data are presented as mean±SEM.*

**Significant at p<0.05.*

Furthermore, a significant interaction between genotype and session suggested that while the total number of trials initiated increased from the first to the last session for *Igf2*-P0 KO mice and WT controls, the *Igf2*-Null KO mice displayed a reduction ($F_{2,51}$= 5.94, p< 0.01). There was an overall increase between the first and the last session of the single nose-poke phase in the number of nose-pokes emitted in response to the initiation stimulus, per trial (within-subject test; $F_{1,51}$= 14.50, p< 0.001) and a significant interaction between the genotype group and session suggested that the number of initiation nose-pokes was greater by *Igf2*-Null KO mice than among subjects of the other genotypes during the last session ($F_{2,51}$= 4.53, p< 0.05). However, a repeated-measures analysis did not report any differences in the number

panel-pushes made per trial between the first and last session of the single nose-poke phase (within-subject test; $F_{2,51}$= 0.51, n.s.) and no differences were shown in the number of panel-pushes per trial between genotype (between-subject test; $F_{2,51}$= 0.23, n.s.). Finally, although no difference was observed between WT-littermate controls on most of the main parameters, the WT controls of the *Igf2*-Null line did exhibit relatively higher number of INIT nose-pokes and panel-pushes per trial than WT controls of the *Igf2*-P0 line. Further, this was particularly noticeable during the final session (see Appendix 4.3).

6.3.2.2 Acquisition of the Go signal trial phase

During sessions where Go signal trials were introduced, mice were only rewarded with a delivery of the reinforcer when they made a correct nose-poke to the light stimulus presented in the aperture on the right side of the response array following a correct response to the initiation stimulus. The duration of the Go signal (Go-LH) was initially set at 60 sec, but was decreased as the mice progressed through this acquisition phase independently for each individual subject. Mice of all genotypes progressed through the training phase within 25 sessions on average, and no significant difference was noted between genotypes (between-subject test; $F_{2,51}$= 2.68, n.s.).

There was no difference found in the percentage of correct response to the Go signal (Go-Correct %) between the first and the last session of the double nose-poke acquisition phase (Figure 6.4A, within-subject test; $F_{1,51}$= 0.07, n.s.), nor was there any difference found between mice of different genotypes ($F_{2,51}$= 1.80, n.s.). On the other hand, there was a significant reduction in the correct response reaction time (Go-Correct RT) between the first (M= 2.51 sec) and the last session (M= 0.70 sec) of the double nose-poke phase (Figure 6.4B, within-subject test; $F_{1,51}$= 41.37, p< 0.001), although no difference was observed between genotypes ($F_{2,51}$= 0.29, n.s.). The overall percentage of trials completed by subjects did not differ between the sessions (within-subject test; $F_{1,51}$= 0.18, n.s.) or between genotypes (between-subject test; $F_{2,51}$= 0.89, n.s.), however there was a significant increase noted in the number of trials initiated in the final session relative to the first session (within-subject test; $F_{1,51}$= 2.06, n.s.). No difference was found in the number of either INIT nose-pokes/per trial (within-subject test; $F_{1,51}$= 0.07, n.s.), Go nose-pokes/per trial (within-

subject test; $F_{1,51}$= 0.41, n.s.), total nose-pokes/ per trial ($F_{1,51}$= 0.65, n.s.) or panel-pushes/per trial (within-subject test; $F_{1,51}$= 0.13, n.s.) across sessions. Similarly, there was no effect of genotype for any of the above parameters; (between-subject test) initiation nose-pokes/per trial (between-subject test): $F_{2,51}$= 0.53, n.s., Go nose-pokes/per trial (between-subject test): $F_{2,51}$= 2.72, n.s., total number of nose-pokes (between-subject test): $F_{2,51}$= 1.54, n.s., and panel-pushes/per trial (between-subject test): $F_{2,51}$= 0.58, n.s., respectively. No difference was noted between the separate WT-control littermates of the two mutant lines, summary of these results is shown in Appendix 4.4.

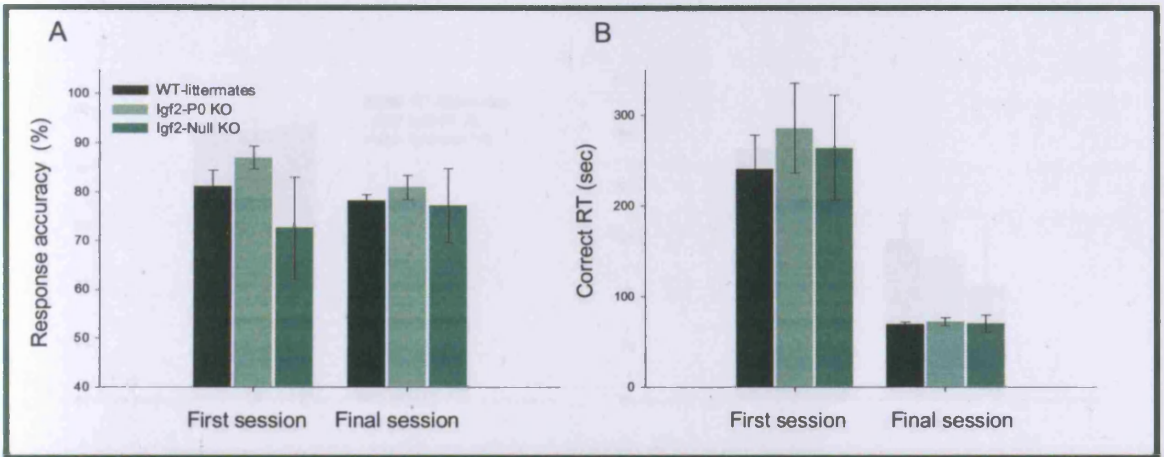

Figure 6.4: *Acquisition of the Go-trial phase. The performance of subjects during at the first and final sessions of the acquisition of the Go-trials, in regards of response accuracy* ***(A)*** *and reaction time* ***(B)****. Data are presented as mean±SEM.*

6.3.2.3 Acquisition of the Stop signal trials phase

Once the mice had acquired the double nose-poke training phase of the SSRTT, they progressed to the next stage of the task acquisition where a burst of white noise representing the stop signal tone, was introduced. At this stage, the Stop-LH was set shorter than the Go-LH, but was increased in stages of 0.1-sec as the subjects progressed onwards. All subjects acquired the Stop signal trial training phase in 10 sessions, although no difference was found in the number of sessions required for the acquisition of this training phase between genotypes (between-subject test); $F_{2,51}$= 0.14, n.s.). A significant reduction was noted in both the percentage of correct stops (Stop-correct %) (Figure 6.5A, within-subject test; $F_{1,51}$= 9.00, p< 0.005) as well as in the reaction time of stopping (SSRT) (Figure 6.5B, within-subject test; $F_{1,51}$=

31.57, $p< 0.001$), from the first session to the last session, as the Stop-LH, the time the mice had to wait for reward delivery, increased. However, no difference was found between genotypes on either measure (between-subject tests; Stop-correct %: $F_{2,51}$= 0.24, n.s.; SSRT: $F_{2,51}$= 0.13, n.s.). Furthermore, there was no difference in the % Go-Correct (within-subject test; $F_{1,51}$= 1.92, n.s.) or in Go-correct RT (within-subject test; $F_{1,51}$= 0.06, n.s.) between the first and final session of the Stop signal trials. In similar vein, both the Go-correct % and Go-correct RT measures were equivalent across different genotype (between-subject tests; Go-correct %: $F_{2,51}$= 0.29, n.s.; Go-RT: $F_{2,51}$= 0.18, n.s.).

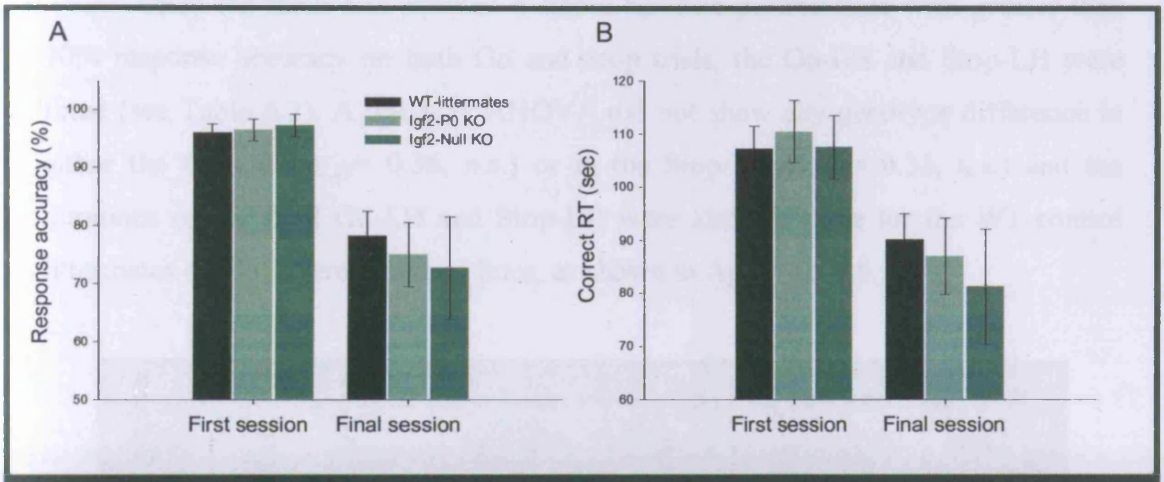

Figure 6.5: *Acquisition of the Stop signal trial phase. The performance of subjects during at the first and final sessions of the acquisition of the stop signal trials, in regard of response accuracy* ***(A)*** *and reaction time of correct Go-responses* ***(B)****. Data are presented as mean values along with SEM.*

The percentage of trials completed by subjects did not differ between the first and final session of the Stop signal trial stage of the SSRTT acquisition phase (within-subject test; $F_{1,51}$= 1.33, n.s.), however there was a significant reduction in total number of trials initiated (within-subject test; $F_{1,51}$= 5.79, $p< 0.05$), but trial numbers for all genotypes still remained >80 out of possible 100/per session. No differences were observed on these measures between genotypes (between-subject tests; % trials-completed: $F_{2,51}$= 0.56, n.s.; trials-initiated: $F_{2,51}$= 1.41, n.s.). Moreover, while there was no difference found between sessions in the number of initiation nose-pokes emitted per trial (within-subject test; $F_{1,51}$= 1.97, n.s.), there was a significant increase with regard to the number Go nose-pokes made per trial (within-subject test; $F_{1,51}$= 4.48, $p< 0.05$). In contrast, there was no difference in the total number of nose-pokes

emitted per trial (within-subject test; $F_{1,51}$= 1.63, n.s.) or the number of panel-pushes (within-subject test; $F_{1,51}$= 1.04, n.s.), and performance on all of these measures was equivalent between genotypes; INIT nose-poke/per trials (between-subject test): $F_{2,51}$= 1.37, n.s., Go nose-pokes/per trial (between-subject test): $F_{2,51}$= 1.47, n.s., total nose-pokes/per trial (between-subject test): $F_{2,51}$= 3.52, n.s. and panel-pushes/per trial (between-subject test): $F_{2,51}$= 1.07, n.s.. Finally, there was no difference found on any the measures between the separate mutant line WT controls (Appendix 4.5).

6.3.3 SSRTT performance at baseline

Once the mice had attained a stable baseline performance with greater than 70% response accuracy on both Go and Stop trials, the Go-LH and Stop-LH were fixed (see Table 6.2). A one-way ANOVA did not show any genotype difference in either the Go-LH ($F_{2,51}$= 0.36, n.s.) or in the Stop-LH ($F_{2,51}$= 0.33, n.s.) and the durations of the final Go-LH and Stop-LH were also the same for the WT-control littermates of the different mutant lines, as shown in Appendix 4.6.

Table 6.2: *SSRTT baseline performance. The results of a one-way ANOVA of several behavioural measures at SSRTT baseline performance, between mice of different genotypes. No significant difference was found on any of the measures at $p<0.05$.*

Behavioural Parameters	Mean ± SE		
	Igf2-P0	Igf2-Null	WT-controls
Go-LH (sec)	0.92 ± 0.03	0.94 ± 0.05	0.94 ± 0.02
Stop-LH (sec)	0.72 ± 0.02	0.71 ± 0.04	0.73 ± 0.01
Trials completed %	73.2 ± 1.2	70.3 ± 3.3	72.4 ± 1.8
No of trials initiated	97.2 ± 0.9	94.4 ± 3.1	98.9 ± 0.4
INIT nose-pokes/per trial	0.84 ± 0.02	0.81 ± 0.04	0.87± 0.02
Go nose-pokes/per trial	0.75 ± 0.02	0.66 ± 0.04	0.74 ± 0.03
Total nose-pokes/per trial	1.60 ± 0.04	1.47 ± 0.07	1.61 ± 0.04
Panel-pushes/per trial	0.88 ± 0.04	0.82 ± 0.06	0.80 ± 0.2

Baseline performance was calculated by averaging parameter values across all sessions which preceeded each of the SSD manipulation session. The results did not show any differences between genotypes in the percentage of correct Stop-trials executed (Figure 6.6A, one-way ANOVA; $F_{2,51}$= 0.50, n.s.) or in the length of the SSRT (figure 6.6B, one-way ANOVA; $F_{2,51}$= 0.13, n.s.). Further, neither response

accuracy (one-way ANOVA; $F_{2,51}$= 0.02, n.s.) nor correct-RT (one-way ANOVA; $F_{2,51}$= 0.30, n.s.) during Go signal trials varied between subjects of different genotypes at baseline performance (as shown in figures 6.6A and 6.6B). Interestingly however, the reaction time of an incorrect Go-response during Stop signal trials was significantly lower than the reaction time of a correct Go-response during Go signal trials (within-subject test; $F_{1,51}$= 50.52, p< 0.001). This acceleration in response time could possibly reflect a reduction in impulse control when making an erroneous response (see Logan, 1984). Equivalent performance was also observed in the percentage of trials completed by subjects (one-way ANOVA; $F_{2,51}$= 0.27, n.s.), as well as in the overall number of trials initiated (one-way ANOVA; $F_{2,51}$= 0.27, n.s.). As shown in Table 6.2, mice of different genotypes did not display any behavioural difference in terms of; number of INIT nose-pokes, Go nose-pokes or total nose-pokes, per trial at baseline performance. In addition, there was no difference in the number of panel-pushes emitted per trial between subjects of different genotypes. A separate analysis which was carried out on these measures, between the separate mutant lines WT-controls did not show any difference, as summarized in Appendix 4.7.

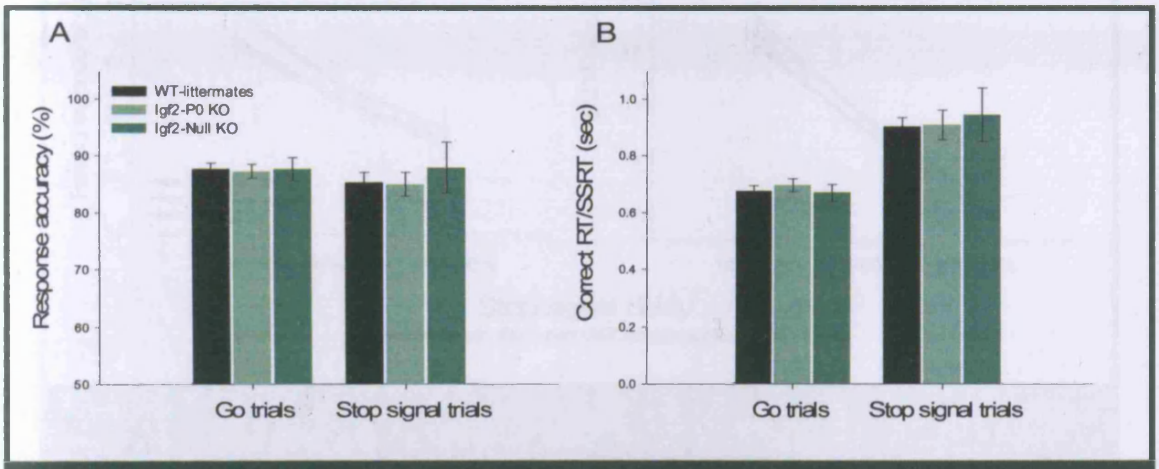

Figure 6.6: *SSRTT baseline performance. The performance of subjects at SSRT baseline level, in terms of response accuracy* ***(A)*** *and reaction time* ***(B)****, at both Go trials and Stop signal trials. Data are presented as mean values along with SEM.*

There were no significant effects of genotype on magazine latency (one-way ANOVA; $F_{2,51}$= 3.16, n.s.) or eating duration (one-way ANOVA; $F_{2,51}$= 0.01, n.s). The latency to respond to the initiation signal was also the same between mice of

different genotypes ($F_{2,51}$= 3.16, n.s.). Finally, no difference was found on the magazine or eating duration parameters (see Appendix 4.8), between the separate mutant line WT controls In common, there was no difference in the initiation response latency between the WT controls of the separate mutant lines ($F_{1,28}$= 0.03, n.s.).

6.3.4 The effects of varying the Stop signal delay on SSRTT performance

Once a stable baseline performance was reached, the response control capability of the subjects was further investigated by varying the position of the SSD. The subjects completed the SSRTT within 40 sessions from reaching baseline performance, and the number sessions required to complete the SSRTT was equivalent across genotypes (one-way ANOVA;$F_{2,51}$= 0.35, n.s.). Increasing the SSD resulted in a decline in the accuracy of the stopping response (Figure 6.7A, within-subject test; $F_{2,102}$=67.56, p< 0.001), as well as a reduction in the SSRT (Figure 6.7B, within-subject test; $F_{2,102}$=67.56, p< 0.001).

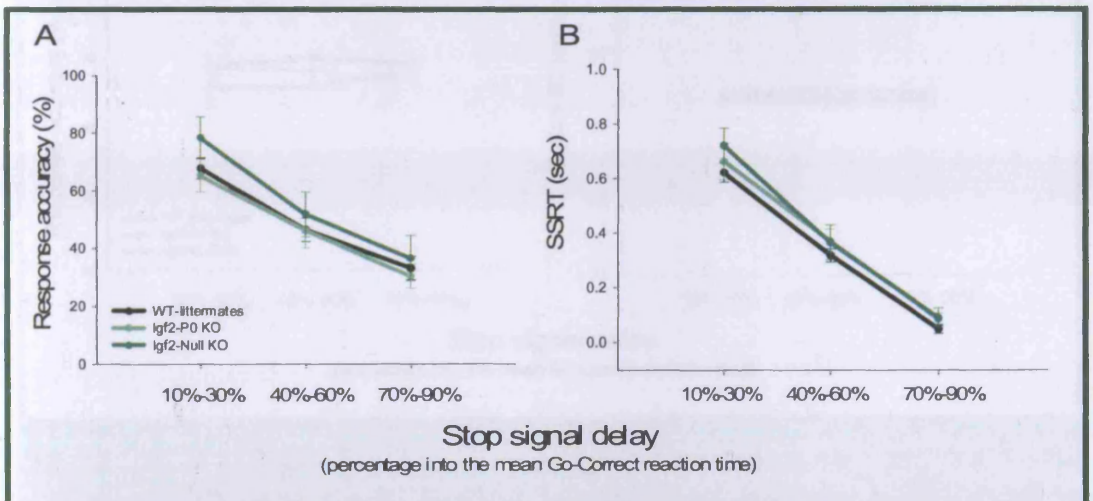

Figure 6.7: *Stop-performanc at varying SSD. The effects of varying the Stop signal delay (from 10% of mean Go-RT to 90% of Go-RT) during Stop signal trials on response accuracy* ***(A)*** *and SSRT* ***(B)****, across genotypes. Data are presented as mean values with SEM.*

A one-way ANOVA did not report any difference between genotypes on either the percentage of correct Stop responses ($F_{2,51}$= 0.33, n.s.) or for SSRT ($F_{2,51}$= 0.56, n.s.), when the Stop signal was presented midway in the mean Go reaction time, a point where the capacity for response inhibition would have been most susceptible. In

sessions where the Stop signal was implemented earlier or later during the Go LH, the results showed no difference at either Go or Stop trials (see Appendix 4.9). Altering the SSD did not affect the percentage of correct Go-responses (Figure 6.8A, within-subject test; $F_{2,102}$= 0.43, n.s.) or the reaction time of correct Go-responses (Figure 6.8B, $F_{2,102}$= 0.08, n.s.) and there were no significant genotype differences found in response accuracy at Go-trials (between-subject test; $F_{2,51}$= 1.96, n.s.) or in the reaction time of the Go-response (between-subject test; $F_{2,51}$= 0.49, n.s.). The percentage of trials completed by subjects did not differ between mice of different genotypes (between-subject; $F_{2,51}$= 0.53, n.s.), and the overall number of trials initiated was also equivalent (between-subject test; $F_{2,51}$= 0.28, n.s.). Also, on a separate analysis of the effects of varying the SSD on the main SSRTT parameters between WT-control littermates of different lines, results did not show any difference as shown in Appendix 4.10.

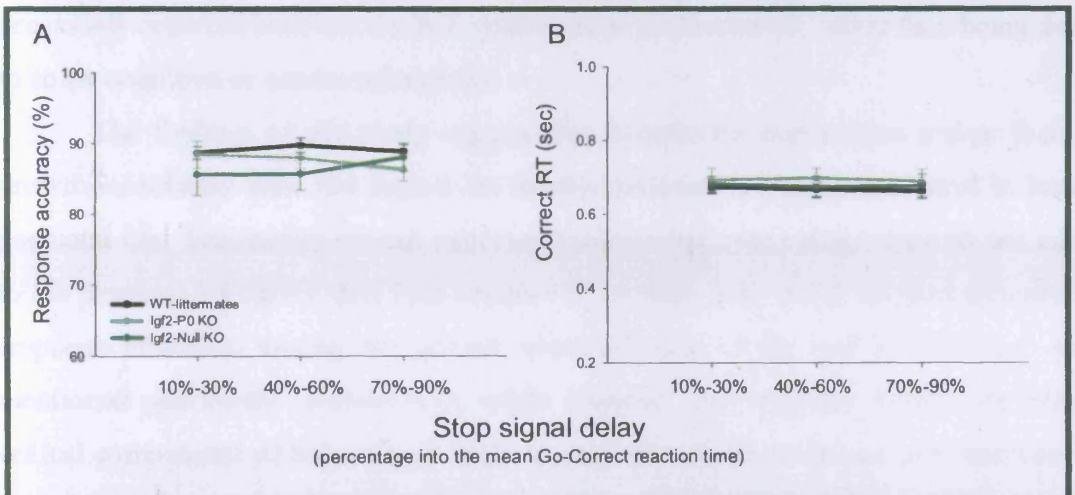

Figure 6.8: *Go-performance at varying SDD. The performance of subjects with varying the Stop signal delay (from 10% of mean Go RT to 90% of Go RT) on response accuracy on Go-trials* ***(A)*** *and Go reaction time of correct responses* ***(B)*** *on the SSRTT, across genotypes. Data are presented as mean values with SEM.*

6.4. Discussion

The SSRTT was successfully carried out on mice of the foetally growth deficient *Igf2*-KO lines and their WT-littermate controls. The majority of subjects showed a steady progress throughout task acquisition and completed the SSRTT within the predetermined time window (i.e. 125 sessions). The relatively large decline

in response accuracy during Stop signal trials along with the lowered reaction time of correct responses during both Go- and Stop-trials reflects the relative sensitivity of the performance of subjects to the dependency of the SSD. The main findings of the present study showed that there was no noticeable difference detected on the measure of response accuracy among *Igf2*-KO mice, in either Go- or Stop-responses, in comparison to the WT littermates. Moreover, the results demonstrated an equivalent SSRT (and Go reaction time for that matter) across mice of different genotypes. The *Igf2*-KO mice acquired the SSRTT at similar rate as their WT-littermate controls, and did not differ on any of the other behavioural measures. Of notice, during the single nose-poke phase of the SSRTT acquisition, the WT littermates of the *Igf2*-Null KO line made fewer nose-pokes in response to the initiation signal per trial, as well panel-pushes, relative to the WT controls of the *Igf2*-P0 KO line. However, since both of these behavioural measures rely heavily on locomotor ability, this difference could likely be accounted for by the difference in body weight (and size) that was previously reported between the WT-control lines in Chapter III, rather than being due to some cognitive or emotional aspects.

The findings of this study suggest that intrauterine malnutrition and/or foetal growth retardation does not impact on murine measures of response control in later postnatal life. The results are not necessarily surprising even though they do not add to the previous 5-CSRRT data (see Chapter V, sections 5.4), which showed enhanced response inhibition among the growth restricted mice of the *Igf2*-P0 KO line. As mentioned previously (section 6.1), while impulse- and response control are both critical components of behavioural inhibition (albeit unlikely to be the only ones) and have both been strongly associated with a diagnosis of ADHD (Winstanley et al., 2006), the behavioural phenotypes of the two inhibitory mechanisms are quite distinct; in terms that one involves cognitive inhibition whereas the other relies more on motor inhibition (Logan and Cowan, 1984).

Furthermore, as previously mentioned in the introduction of this chapter, there is no reason to assume that both impulse- and response control are modulated by the same neurobiological substrates, even if they are likely to overlap to some extent. In fact, while catecholaminergic (noradrenaline and dopamine) and serotonergic (5-HT) neurotransmitter systems have been demonstrated to play a critical role in the functions of behavioural inhibition in the 5-CSRTT (see Robbins, 2002 for review), the evidence suggests that the serotonergic influence on SSRTT performance is only

slight (Robbins, 2007). For instance, numerous studies have reported an increase in the number of premature nose-pokes performed in the 5-CSRTT following 5-HT depletion (Carli and Samanin, 2000; Harrison, Everitt and Robbins, 1997; Koskinen et al. 2000; Koskinen and Sirvio, 2000), whereas studies using selective 5-HT re-uptake inhibitors and tryptophan depletion have only reported minor if any effects on response control in the SSRTT (Chamberlain et al., 2006; Clark et al., 2005; Eagle et al., 2009). However, numerous studies have shown that the dopaminergic and noradrenergic systems may play significant roles in controlling SSRTT performance (Eagle et al., 2007; Turner et al., 2004; Chamberlain et al., 2009; Robinson et al., 2008), as might be expected in a task sensitive to ADHD (Prince, 2008). In context of this pharmacological data and the results from the current study, it could be proposed that early programming by intrauterine under-nutrition might have either directly or indirectly, influenced the developmental trajectory of a brain system particularly important for mediating impulse control in the 5-CSRTT (the 5-HT system for example) and resulted in altered neurochemical functions in the 5-HT pathways in later postnatal life. These changes could in turn have improved the impulse inhibition of the *Igf2*-P0-KO mice without affecting SSRTT performance. Still, despite the feasibility of this account based on the dissociation of the two response inhibition phenotypes observed, further research into the multiple 5-HT neurobiological substrates that might modulate impulse inhibition (i.e. 5-HT biosynthesis, -reuptake mechanisms, receptor subtype expression etc.) is warranted in order to provide more conclusive evidence for this possibility.

In conclusion, there were no significant differences observed among the *Igf2*-KO mice and WT-controls on SSRTT performance. The SSRTT assays a different form of behavioural inhibition than the 5-CSRTT, and highlights the possibility that the changes shown in the *Igf2*-P0 KO mouse model are particular to cognitive inhibition, rather than broader behavioural inhibition. As such, these differences in behavioural phenotypes in *Igf2*-P0 KO mice could stem from an early developmental reprogramming of the 5-HT system, although changes in noradrenergic and/or dopaminergic transmissions is also a possibility.

6.4.1 Summary of key results from Chapter VI

- *Igf2*-KO mice did not show any differences from WT controls in either Go- or Stop-response accuracy during the SSRTT.
- The SSRT was indistinguishable between *Igf2*-KO mice and WT control at baseline and under conditions of varying SSD.
- *Igf2*-KO mice acquired the SSRTT at similar rate as their WT controls, and did not differ on any of the other behavioural measures.

Chapter VII

Investigating the Molecular Substrates of the Heightened Stress/Anxiety-Related Phenotypes and Impulse Control among Mice of the Igf2-P0 Knockout Mice: A Role for Hippocampal GABAergic and Serotonergic Systems

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7.1 Introduction

Among the main findings from the series of behavioural experiments reported in the preceding chapters was that the *Igf2*-P0 KO mice exhibited heightened stress response and anxiety-related phenotypes, in comparison to wild-type (WT) control littermates. The increased stress response among *Igf2*-P0 KO mice was indicated by a significantly greater whole body startle reaction to acoustic stimuli, whilst an increase in anxiety-related behaviours was observed across a number of ethological based conflict tests in this group. The aim of this chapter is to assay the expression of key neural/molecular substrates known to be vulnerable to early environmental insults, and which have been demonstrated to have a role in stress/anxiety-related behaviour with a view to beginning to understand the molecular mechanisms underlying the behavioural differences reported in previous chapters.

The hippocampal circuitry plays an integral part in stress/anxiety responses (Chapter IV, section 4.1), predominantly via the neurotransmitters GABA and serotonin (5-HT) and their different receptor subtypes (Kim and Gorman, 2005; Millan, 2003). Accordingly, stress- and/or anxiety-related psychiatric disorders are frequently characterized by dysfunctions of the GABA- and 5-HTergic systems (Millan, 2003; Graeff, Guimarães, De Andrade and Deakin, 1996; Kalueff and Nutt, 2006, respectively), and these neurotransmitter systems have also been found to be affected by the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Kirby et al., 2008; Leonard, 2005). Furthermore, in parallel to the aforementioned findings of a marked hippocampal susceptibility to nutritional programming during early development (see Chapter I, section 1.3.2), there is a strong body of evidence that GABA- and 5-HTergic neurotransmission is attenuated by poor intrauterine nutrient availability (Steiger, Alexander, Galler, Farb, and Russek 2003; Fiacco, Fitzgerald,

Blatt, Rosene, and Galler, 1998; Mokler, Galler and Morgane, 2003; Mokler, Bronzino, Galler and Morgane, 1999).

GABA is the major inhibitory neurotransmitter of the central nervous system (CNS) and is therefore involved in a number of different physiological actions. The usage of anxiolytic drugs, such as the benzodiazepines, which modulate GABA function, have suggested a key role for the GABAergic system in the control of anxiety-related behaviours. Benzodiazepines act to potentiate the inhibitory effects of GABA via modulation of the $GABA_A$ receptor by binding at different molecular sites in the receptor complex. The benzodiazepine (BZ) type-I receptor site (containing the $GABA_{\alpha 1}$ receptor subunit) has been suggested to be involved in sedation but the BZ type-2 receptor site which encompasses the $GABA_{\alpha 2}$ and $GABA_{\alpha 3}$ subunits, has been found to play a key role in modulating anxiety (Atack, 2005), and moreover the expression of these receptor subunits is highly enriched in the hippocampus (Möhler, Fritschy and Rudolph, 2002). The expression of $GABA_{\alpha 2}$ and $GABA_{\alpha 3}$ receptor subunits has previously been shown to be influenced by intrauterine nutrition in a rat model (Steiger, Galler, Farb and Russek, 2002), implicating them as candidate molecular mediators for the previously described behavioural effects.

In terms of the serotonergic system, the $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A}$ receptor subtypes have been shown to play a pivotal role in modulating anxiety-related behaviour, although the manner in which these receptors function in stress and anxiety remains obscure. For example, down-regulated expression of the $5\text{-}HT_{1A}$ receptor subtype has repeatedly been noted in stress-and/or anxiety-related disorders in both humans (Mann, 1999; Lopez., Chalmers, Little and Watson, 1998) and animals (Lopez et al., 1998; Flugge, 1995; McKittrick, Blanchard, Blanchard, McEwen, and Sakai, 1995), and gene deletion of the $5\text{-}HT_{1A}$ receptor in mice leads to a robust anxiogenic phenotype, exemplified by heightened levels of avoidance to a novel unfamiliar environment and related increase in stress response (Toth, 2003). Moreover, a large number of studies have described anxiolytic effects in humans and rodents, following administration $5\text{-}HT_{1A}$ partial or full agonists (De Vry, 1995; Schreiber and De Vry, 1993 and 1992; Higgins, Jones, and Oakley, 1992). There is less of a consensus for the role that the $5\text{-}HT_{2A}$ receptor may have in anxiety-related behaviours. In contrast to the effects of $5\text{-}HT_{1A}$ gene deletion, a $5\text{-}HT_{2A}$ receptor knockout mouse model has recently been reported to suppress avoidant behaviour in a range of approach-avoidance conflict paradigms (Weisstaub et al., 2006). There are discrepancies in the

results of work using 5-HT_{2A} receptor agonist agents which describe anxiogenic effects on rodent behaviour (see Griebel, 1996 for review), whereas other more recent studies demonstrate the reverse (Massé, Áine, Dhonnchadha, Hascoët and Bourin, 2007; Ripoll, Hascoët and Bourin, 2005; Dhonnchadha, Hascoët, Jolliet and Bourin, 2003). The differential results of 5-HT_{2A} receptor agonist studies most probably stem from regionally-specific effects of the 5-HT_{2A} receptor in anxiety-related behaviour. In support of this notion, localized micro-infusion in either the amygdala or periaqueductal gray of the 5-HT_{2A} receptor agonist DOI ((±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane) has been shown to cause an increase in anxiety-related behaviour (Massé, Petit-Demouliere, Dubois, Hascoët, and Bourin, 2008), whereas infusion into the dorsal hippocampus has evoked profound anxiolytic effects. Of particular relevance to this thesis, malnutrition during intrauterine life in rats has been shown to down-regulate 5-HT_{1A} receptor subtype brain expression (Blatt, Chen, Rosene, Volicer and Galler, 1994); however, to the author's knowledge, the same has not been demonstrated for the 5-HT_{2A} receptor. On the basis of this evidence it is reasonable to assume that the heightened stress- and anxiety-related phenotypes of *Igf2*-P0 KO mice might stem from reduced expression of GABAergic and/or 5-HTergic receptors in the hippocampus.

Another key finding in *Igf2*-P0 KO mice was enhanced impulse control in the 5-choice serial reaction time task (5-CSRTT), as indexed by the significantly reduced number of premature nose-pokes performed at baseline and during the session in which the inter-trial interval was lengthened (see Chapter V). This result is of considerable interest, as there is evidence for greater behavioural inhibition associated with anxiety disorders in humans (see Colder and O'Connor, 2004; Turner, Beidel and Wolff, 1996 for review). However, similar findings have not been reported in any of the previous animal models of intrauterine under-nutrition. In fact, studies have noted a decrease in response inhibition among intrauterine malnourished rats during a simple reward-conditioning paradigm (Tonkiss et al., 1990b; Smart et al., 1973). Nevertheless, it is feasible that the improved impulse control of *Igf2*-P0 KO mice might originate as a result of the increase in anxiety and there is a substantial amount of both behavioural and biological data supportive of such a relationship, see section 8.2 for discussion.

The 5-HT system has been strongly implicated in behavioural inhibition in both humans and animals (Winstanley et al., 2006). Numerous animal studies have

underlined the importance of 5-HT for impulse control in the 5-CSRTT (Robbins, 2002) where attenuations in 5-HT by global forebrain lesion (Harrison et al., 1997a) or agonism of the 5-HT_{1A} receptor (which reduces 5-HT release, Carli and Samanin, 2000) increase the number of premature nose-pokes on the 5-CSRTT. Moreover, 5-HT efflux, as measured in the medial prefrontal cortex using *in vivo* microdialysis during the 5-CSRTT, was shown to be increased in situations where greater impulsivity was induced (Dalley, Theobald, Eagle, Passetti and Robbins, 2002) further indicating the link between 5-HT and impulsivity in the 5-CSRTT. The complexity of the 5-HT system, which includes over 15 different types of receptors in the mammalian system (Robinson et al., 2008), means that it can exert both excitatory and inhibitory actions on serotonergic neurons, and on their dopamingergic and glutamatergic targets. For example, a recent study demonstrated a significant reduction in premature responding on the 5-CSRTT following microinfusion of the 5-HT_{2A} antagonist M100907 into the ventral striatum (i.e. nucleus accumbens), whereas infusion of SB242084, a 5-HT_{2C} receptor subtype antagonist, heightened this parameter of impulse control (Robinson et al., 2008). Conversely, administration of either SB242084 or M100907 in the medial prefrontal cortex (i.e. prelimbic cortex) did not produce any effect on impulse control during the 5-CSRTT, highlighting the functional importance of the striatum for behavioural inhibition and regional specificity of this response. In addition, this functional dissociation between 5-HT_2 receptor subtypes has been demonstrated in previous studies (Higgins, Enderlin, Haman and Fletcher, 2003; Winstanley et al., 2003). Hence, the enhanced impulse control displayed by the *Igf2*-P0 KO mice during the 5-CRSTT could be due to alterations in the expression of these 5-HT receptor subtypes in specific regions of the brain, most notably the striatum.

In the present study, a quantitative real-time polymerase chain reaction (qPCR) analysis was performed to investigate expression levels of several of the target receptor subtypes which have been implicated with anxiogenic behaviour and impulse control. In relation to the heightened levels of stress response and anxiety-related behaviour in the *Igf2*-P0 KO mice, the expression of $GABA_{\alpha 2}$, $GABA_{\alpha 3}$, and 5-HT_{1A} and 5-HT_{2A} receptor subtypes was examined in the hippocampal region, whereas striatal expression of 5-HT_{2A} and 5-HT_{2C} receptor subtypes was examined for potential involvement in the enhanced impulse control observed during 5-CSRTT. Additionally, the expression of the two different splice variants of the $5HT_{2C}$ receptor

(see Hannon and Hoyer, 2008), the functional 5-HT_{2CLong} and nonfunctional 5-$HT_{2CShort}$, and their ratio, were examined. As the major behavioural effects that have been found so far (see Chapters III-VI) relate to the *Igf2*-P0 KO mice, analysis was limited to these mice and their own WT-littermate controls (i.e. WT-P0 littermates).

7.2 Material and Method

7.2.1 Real-time qPCR

Cerebral tissue was collected from a total of 16 mice from different litters of *Igf2-P0* KO and their WT-control littermates; consisting of eight subjects in each genotype group. All subjects (6-8 months old, age-matched between genotypes) were culled by cervical dislocation and the brain rapidly removed, The cerebral structures dissected and obtained for the purpose of present investigation included; the hippocampus, hypothalamus and striatum (see Chapter II, section 2.7). RNA was extracted from both hemispheres and prepared in accordance to the protocol previously described in Chapter II (section 2.8). Total extracted RNA was synthesized into cDNA using a Sprint^{TM} RT Complete Products Kit (Clontech, Mountain View, CA) along with the accompanying Poly-dT protocol (as outlined in Chapter II, section 2.8.5). Real-time qPCR analysis was carried out on the cDNA samples in order to determine level of gene expression in the aforementioned brain structures (see Chapter II, section 2.8.7). All of the reactions were arranged in triplicate (in effort to minimize pipetting errors) and a Corbett CAS-1200 robotic bench top instrument (Corbett Life Science) enabled automated PCR setup. All primer concentrations were optimized before use (see Chapter II, section 2.8.6). The qPCR was run on a Rotor-Gene^{TM}6000 cycler machine.

The assessment of $GABA_{\alpha 2}$, $GABA_{\alpha 3}$, 5-HT_{1A} and 5-HT_{2A} subunit expression in the hippocampus was of particular importance for the purpose of examining potential molecular targets underlying the stress/anxiety-related phenotypes observed in the *Igf2*-P0 KO mice (Chapter IV). In relation to the heightened impulse control observed among the *Igf2*-P0 KO mice (Chapter V), expression of the 5-HT_{2A} receptor and 5-HT_{2C} receptor subunit variants; 5-$HT_{2C\text{-}long}$ and 5-$HT_{2C\text{-}short}$, was examined in the striatum, as well as the expression ratio between the two variants as an index of alternative splicing (calculated as 5-$HT_{2C\text{-}long}$ / 5-$HT_{2C\text{-}short}$). Expression of the

housekeeping gene 18S, was also determined in each brain region as a control for RNA concentrations (see section 7.2.2.1).

7.2.2 Real-time PCR analysis: 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. Furthermore, the $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments. Derivation of the $2^{-\Delta\Delta Ct}$ equation, including assumptions, experimental design and validation tests have been previously described by VanGuilder et al. (2008), and Livak and Schmittgen (2001).

7.2.2.1 Selection of internal control and calibrator for the $2^{-\Delta\Delta Ct}$ method

Due to the fact that samples vary in size, and thus the amount of RNA obtained, false expression data could be produced by the varying concentration of extracted RNA, or the converted cDNA. Therefore, in order to normalise the PCR reactions for the amount of RNA added to the reverse transcription reaction, an internal gene (otherwise known as house-keeping gene), was required to establish a control. Importantly, the expression of the house-keeping gene needs to be abundant and unaffected by genetic abnormalities or chemical treatments. For these reasons, 18S RNA was selected as a suitable control for the real-time qPCR analysis. The selection of calibrator for the $2^{-\Delta\Delta Ct}$ method was based on the kind of gene expression analysis, and for the current study the most basic design, the untreated control, was used. While using the $2^{-\Delta\Delta Ct}$ method, the data was presented as fold change in gene expression, normalised to the endogenous house-keeping gene and relative to the WT controls. In case of the of the WT control, the $\Delta\Delta Ct$ equals 0 and 2^0 equals 1, whereby the fold change in gene expression relative to the WT controls equals 1, by definition. On the other hand, in case of the mutant sample evaluation of the $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the WT control.

7.2.2.2 Data analysing using the $2^{-\Delta\Delta Ct}$ method

The *Ct* values obtained from the Rotor-Gene™6000 cycler software were imported into Microsoft Excel, which enabled descriptive analysis of the data. The *Ct*

values for the 18s and the target gene rRNAs were averaged across the triplicates for each sample, prior to performance of the ΔCt calculation. Next, the ΔCt value was calculated by subtracting average Ct values of the target genes from the average Ct values of the 18S. The estimated variance from the replicate Ct values is used in the final calculation of relative quantities using standard propagation of error methods. However, due to the fact that the Ct value is exponentially related to the number of copies, for the final calculations the error was estimated by evaluating the $2^{-\Delta\Delta Ct}$term using the adding/subtracting of the standard deviation to/from the $\Delta\Delta C$t values. This results in a range of values that are asymmetrically distributed relative to the average value, as the asymmetric distribution is consequence of converting the results of an exponential process into a linear comparison of amounts. These values are then combined with the values of relative change in the expression of the target gene between the comparison groups and presented as a graph.

7.2.2.3 Statistical analysis of the real-time PCR data

The final stage of the real-time qPCR analysis is to determine the threshold cycle or the Ct value. The Ct value is derived from a log-linear plot of the PCR signal against the cycle number, which depicts the Ct value as an exponential instead of linear term. Hence, the data was converted to a linear form using $2^{-\Delta Ct}$, which then demonstrated the individual variation among replicates with further accuracy. The data obtained was statistically analysed using a one-tailed independent T-test, while a *Benjamini Hochberg* false discovery rate correction testing (Benjamini and Hochberg, 1995) was used to adjust p-values for false positives, due to multiple testing. The $2^{-\Delta\Delta Ct}$ relative method was applied to graphically present the gene expression data, but this method was designed to assess gene expression in a set of samples relative to the expression of the same genes in a control (i.e. wild-type) set of samples.

7.3 Results

7.3.1 Hippocampal expression of GABA- and 5-HTergic receptor subtypes

Expression of GABA and 5-HT receptor mRNA in the hippocampus and hypothalamus was measured to determine if changes in these neurotransmitter systems might account for the increased stress/anxiety effects observed in *Igf2*-P0 KO

mice (see Chapter IV). Real-time qPCR data revealed a significant ~35% down-regulation of the $GABA_{\alpha 3}$ subunit expression in the hippocampus of *Igf2*-P0 KO mice in comparison to their own WT-control littermates (Figure 7.1A, $t(14)=-3.15$, $p<0.01$, significant following *Benjamini-Hochberg* adjustment), whereas no apparent difference was detected in the expression of $GABA_{\alpha 2}$ in this brain region ($t(14)= 0.13$, n.s.). Moreover, there was no variation in $GABA_{\alpha 3}$ expression observed in the hypothalamus ($t(14)= 0.93$, n.s.). These data suggest regional specificity in the alteration of $GABA_{\alpha 3}$ expression in *Igf2*-P0 KO mice.

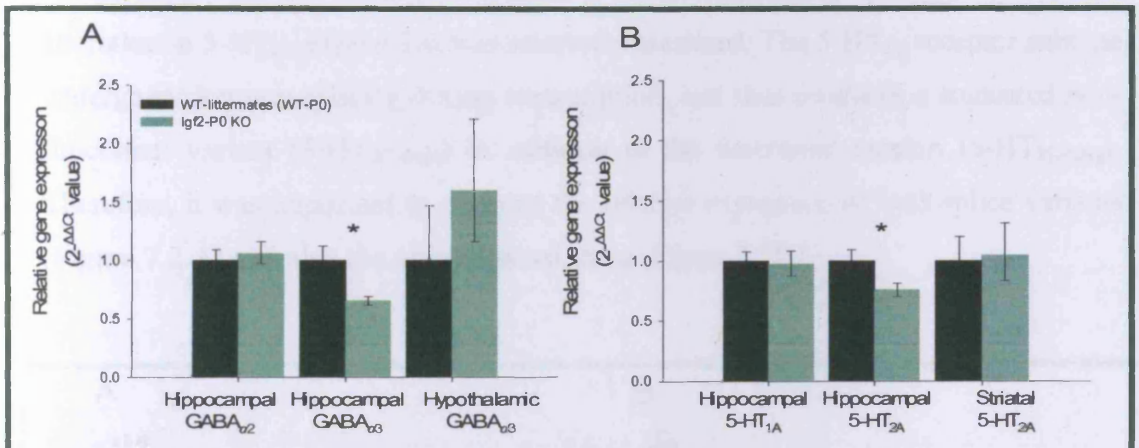

Figure 7.1: *Expression of certain GABA and 5-HT receptor subtypes, implicated with anxiety-related behaviour in the hippocampus and control brain regions (i.e. hypothalamus and striatum).* ***(A)*** *The expression of $GABA_{\alpha 2}$ and $GABA_{\alpha 3}$ receptor subunits in hippocampal and hypothalamic brain regions, between Igf2-P0 KO mice and their WT-littermates.* ***(B)*** *The expression of 5-HT_{1A} and 5-HT_{2A} receptor subtypes in hippocampal and striatal regions, between Igf2-P0 KO mice and their WT-littermates. Data are presented as mean±SEM.*

**Significant at $p<0.05$ following a Benjamini Hochberg correction for multiple testing.*

A similar pattern of effects was observed when gene expression of the 5-HT_{1A} and 5-HT_{2A} receptors was assayed. There was a significant ~24% reduction in 5-HT_{2A} receptor subunit expression in the hippocampus of *Igf2*-P0 KO mice (Figure 7.1B, $t(14)=-2.64$, $p<0.01$, significant following *Benjamini Hochberg* adjustment). In contrast, gene expression of the 5-HT_{1A} receptor in the hippocampus ($t(14)=-0.55$, n.s.), and the 5-HT_{2A} receptor in the striatum ($t(14)= 0.03$, n.s.) did not differ between *Igf2*-P0 KO mice and their WT littermates. Thus, consistent with the previous results, these data further highlight possible dysfunction in the hippocampus as a neural

mechanism that might underlie the altered stress/anxiety behaviour of *Igf2*-P0 KO mice.

7.3.2 Striatal expression of 5-HT$_2$ receptors

In the context of the heightened impulse control exhibited by the *Igf2*-P0 KO mice (Chapter V), striatal mRNA expression of the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor subtypes was assayed. No difference was noted in expression of the 5-HT$_{2A}$ receptor subtype in the striatum (see Figure 7.1A above, $t(14)= 1.61$, n.s.), which in comparison with the reduced expression in the hippocampus suggests that the alteration in 5-HT$_{2A}$ expression was relatively localized. The 5-HT$_{2C}$ receptor subtype undergoes alternate splicing during transcription, and thus produces a truncated non-functional variant (5-HT$_{2C\text{-short}}$) in addition to the functional version (5-HT$_{2C\text{-long}}$). Therefore, it was important to examine the relative expression of both splice variants (Figure 7.2.A) and also the ratio between them (Figure 7.2B).

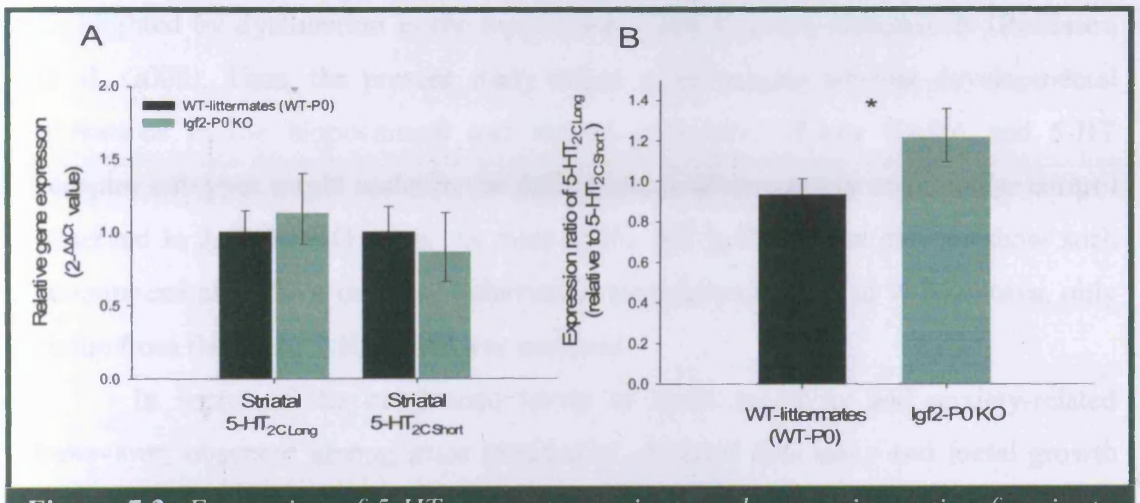

Figure 7.2: *Expression of 5-HT$_{2C}$ receptor variants and expression ratio of variants.* ***(A)*** *Striatal expression of 5-HT$_{2A}$, 5-HT$_{2C\text{-long}}$ and 5-HT$_{2C\text{-short}}$ receptor variants, in Igf2-P0 KO mice and their WT-littermates.* ***(B)*** *The striatal expression ratio of the 5-HT$_{2C\text{-long}}$ receptor variant relative to the short non-functional 5-HT$_{2C\text{-short}}$, in Igf2-P0 mice and their WT-littermates. Data are presented as mean values along with SEM.*

**Significant at $p<0.05$.*

There were no differences in the relative expression between the *Igf2*-P0 KO mice and their WT-littermate controls of either the functional variant of the 5-HT$_{2C}$ receptor subtype (5-HT$_{2C\text{-long}}$, $t(14)= 0.84$, n.s.), or for the non-functional variants (5-

$HT_{2C\text{-}short}$, $t(14)= 0.03$, n.s.). However, calculation of the ratio of expression for the two transcription variants revealed a significant alteration in the splicing of 5-HT_{2C} transcripts between the *Igf2*-P0 KO mice and their WT controls (Figure 7.2B, $t(14)= 2.00$, $p<0.05$). This increased ratio in *Igf2*-P0 KO mice suggests a shift towards greater expression of the functional 5-$HT_{2C\text{-}long}$ transcript and may represent an overall increase in 5-HT_{2C} receptor functionality in the striatum, which in turn may underlie the altered impulse control shown by *Igf2*-P0 KO mice.

7.4 Discussion

Previous research in the field of foetal programming, as a result of altered *in utero* nutrition, has demonstrated the involvement of aberrant alterations in the physiological feedback circuitry of GABA and 5-HT systems, in the pathogenesis of later stress/anxiety-related disorders and there is substantial evidence implicating links between the 5-HT-ergic system and impulse control (see section 7.1). Moreover, alterations in these neurotransmitter systems have shown regional specificity, highlighted by dysfunction in the hippocampus and striatum, respectively (Robinson et al., 2008). Thus, the present study aimed to investigate whether developmental alterations in the hippocampal and striatal expression of key GABA and 5-HT receptor subtypes might underlie the differences in stress/anxiety and impulse control observed in *Igf2*-P0 KO mice. As mice of the full *Igf2* KO line did not show such pronounced alterations on these behavioural parameters relative to WT-controls, only tissue from the *Igf2*-P0 KO mice was analysed.

In terms of the heightened levels of stress reactivity and anxiety-related behaviour, observed among mice exposed to placental deficiency and foetal growth restriction (i.e. the *Igf2*-P0 KO line), the developmental alteration in the hippocampal expression of key GABA and 5-HT receptor subtypes was investigated. Findings from the real-time qPCR analysis revealed a significant reduction in the expression of $GABA_{\alpha 3}$ receptor subunit in the hippocampus, although no difference was noted in $GABA_{\alpha 2}$ mRNA levels. Moreover, the fact that the reduction in $GABA_{\alpha 3}$ expression was absent in the hypothalamus, supports the idea that nutritional insults in early life might exert region-specific effects on gene expression in the brain. This reduction in $GABA_{\alpha 3}$ expression is in line with previous work, indicating the susceptibility of $GABA_{\alpha}$ receptor expression to early malnutrition (Steiger et al., 2002). A regionally-

specific decrease was also observed in the expression of the 5-HT_{2A} receptor subtype in the hippocampus, whereas expression of the 5-HT_{1A} receptor subtype was found to be intact. This result was particularly interesting, on the basis that only the 5-HT_{1A} expression (not 5-HT_{2A}) to the author's knowledge, has previously been shown to be altered by intrauterine malnutrition (Blatt et al., 1994). Moreover, the overall pattern of expression ($GABA_{\alpha 2}$, $GABA_{\alpha 3}$, 5-HT_{1A} and 5-HT_{2A}) suggests quite a specific range of altered gene expression rather than a general non-specific alteration in hippocampal integrity.

Evidence from numerous studies has implicated a strong functional relationship between GABA and 5-HT systems in the pathogenesis of anxiety (see section 7.1), although, as mentioned, there has been particular focus on the 5-HT_{1A} receptor in terms on this relationship. Indeed, reduced 5-HT transmission (mainly via the 5-HT_{1A} receptor), which in turn diminishes $GABA_A$ receptor expression and increases anxiety and benzodiazepine insensitivity in knockout mice deficient of 5-HT_{1A} receptor expression, (Bailey and Toth, 2004), and transient postnatal pharmacological blockade of the 5-HT_{1A} receptor has been shown to produce a long-term increase in anxiety and reduced benzodiazepine sensitivity, but also heightened expression of $GABA_\alpha$ receptor subunits in the prefrontal cortex and the hippocampus (Vinkers, Oosting, van Bogaert, Olivier and Groenink, 2010). However, the major 5HTergic difference observed was in terms of reduced 5-HT_{2A} gene expression in the hippocampus, which has not been previously linked with intrauterine growth restriction (IUGR) but a relationship has been found between the hippocampal function of this receptor and anxiety (Leonard, 2005). As mentioned previously (see section 7.1), there is not a consensus for the effects of manipulations of the 5-HT_{2A} receptor on anxiety where both anxiogenic and anxiolytic behaviours have been observed. The 5-HT_{2A} receptor is widely distributed in the brain (Xu and Pandey, 2000; Burnet, Eastwood, Lacey and Harrison, 1995) and regional-specificity may account for these discrepant results. Thus, the current findings of increased stress/anxiety in *Igf2*-P0 KO mice may well be accounted for by the decreased hippocampal gene expression of the 5-HT_{2A} receptor, but with the contribution of the reduction in $GABA_{\alpha 3}$ expression.

Also noteworthy is the fact that in addition to being strongly implicated with stress-related psychiatric disorders, both the GABA- and 5-HT-ergic systems have been found to be affected by increased HPA axis activity. Secretion of the stress-

regulating corticotropin releasing hormone (CRH) has, for instance, been demonstrated to modulate the transmission of dorsal Raphé 5-HT neurons directly through the CRH_{R2} receptor, as well as indirectly via presynaptic GABA activity (Kirby et al., 2008). In addition, administration of either dexamethasone or natural glucocorticoids has also been found to modulate 5-HT_{1A} and 5-HT_{2A} receptor expression, via glucocorticoid receptors (Leonard, 2005). However, in context of the findings from current study, it should be considered whether this modulating influence of HPA axis function upon the reciprocal interactions of the GABA and 5-HT systems in the dorsal Raphé nuclei, could be broadened to other brain regions (such as the hippocampus) and include other receptor subtypes of these neurotransmitters.

In relation to the heightened impulse control observed in the *Igf2*-P0 KO mice (Chapter V), real-time qPCR analysis did not reveal any difference in the expression level of 5-HT_{2A} receptor subtype in the striatum and although no difference was noted in the striatal expression of either splice variant of the 5-HT_{2C} receptor subtype (i.e. the 5-$HT_{2C\text{-}long}$ and 5-$HT_{2C\text{-}short}$), between the *Igf2*-P0 KO mice and their WT-littermate controls, the results did reveal an alteration in post-transcriptional modification of the 5-HT_{2C} receptor. This was demonstrated by a significant increase in the expression ratio, in terms that the balance between the splice variants was shifted towards the functional 5-$HT_{2C\text{-}long}$ variant in the *Igf2*-P0 KO mice and away from the truncated, non-functional shorter transcript. That the IUGR *Igf2*-P0 KO mice show an altered pattern of 5-HT_{2C} editing is consistent with a previous study where early life stress was also found to lead to altered RNA editing of the 5-HT_{2C} receptor in later life (Bhansali, Dunning, Singer, David, and Schmauss, 2007), and moreover, there is evidence that growth retardation by deletion of an imprinted gene control region (Prader-Willi syndrome, PWS-$IC^{+/-}$ mice) also leads to aberrant editing patterns of 5-HT_{2C} via a snoRNA mediated pathway (Doe et al., 2009). Furthermore, when assessed in the 5-CSRTT, PWS-$IC^{+/-}$ mice were found have attenuated 5-HT_{2C} receptor-mediated impulse control following administration of the specific 5-HT_{2C} antagonist, SB242084, suggesting that altered editing of this receptor has significant functional consequences.

As mentioned previously, there is considerable evidence linking the 5-HTergic system and impulsive behaviour, especially in terms of 5-CSRTT performance. Global forebrain depletion of 5-HT leads to an increase in premature

responding (Harrison et al., 1997a), as does selective depletion of 5-HT in the dorsal Raphé nuclei, which primarily project to the striatum and neocortex (Harrison et al., 1997b). Moreover, administration of SB242084, a specific 5-HT_{2C} antagonist, also increases impulsivity in this task (Winstanley et al., 2003; Doe et al., 2009) suggesting that 5-HT may act via this receptor to control this form of behaviour, possibly by inhibitory modulation of dopamine release from both striatal and ventral tegmental area neurons (Bubar and Cunningham, 2006; Porras et al., 2002; Di Matteo, Blasi, Di Giulio and Esposito, 2001; Gobert and Millan, 1999). Indeed, decreased dopamine levels have repeatedly been reported to attenuate impulse control during the 5-CSRTT (see Robbins, 2002 for review). For example, a dose-dependent increase in impulsive responding (i.e. premature nose-pokes) is noted following striatal infusion of the drug D-amphetamine (Cole and Robbins, 1987), whereas dopamine depletion in the nucleus accumbens of the striatum seems to attenuate this effect (Cole and Robbins, 1989). The pattern of effects observed in *Igf2*-P0 mice of increased response control (decreased impulsivity) and it is highly probable that an increase in the functional form of the 5-HT_{2C} receptor could increase the binding efficacy of 5-HT, suppress forebrain dopamine release, and in turn improve impulse control.

Intriguingly, in recent years there has been accumulating body of work for the differential splicing of the 5-HT_{2C} receptor RNA in various psychiatric disorders, including schizophrenia (Sodhi, Burnet, Makoff, Kerwin, and Harrison, 2001), depression (Dracheva, Chin and Haroutunian, 2007; Iwamoto, Nakatani, Bundo, Yoshikawa and Kato, 2005) and in people of increased risk of suicide (Gurevich et al., 2002; Dracheva et al., 2008), conditions with a high risk of altered response control. Furthermore, while there have still not been any studies directly showing alternate splicing of the 5-HT_{2C} receptor variants in stress- or anxiety-related disorders (although the links with depression and suicide are quite persuasive in terms of increased neuroticism in these conditions), differences in post-transcriptional 5-HT_{2C} editing have been described between anxiogenic rat strains (i.e. BALB/cJ and DBA/2J) and a non-anxiogenic strain (i.e. C57BL/6J, Hackler, Airey, Shannon, Sodhi, and Sanders-Bush, 2006), suggesting that this altered editing may also underlie some of the stress/anxiety phenotypes demonstrated by *Igf2*-P0 KO mice.

Thus, the findings from the present study demonstrated for the first time the long-term effects of foetal exposure to placental deficiency and growth restriction on gene expression in the brain, by using targeted knockout of the promoters of the

imprinted *Igf2* gene. These findings shed an important light on exactly how the developmental trajectories of the CNS are affected by early life nutrition, as well as its implication for later behaviour. However, in the absence of evidence to show altered concentrations of the proteins derived from the mRNA extrapolation of these results needs to be cautionary at this stage. Future analysis of tissue samples by Western blotting techniques, *in situ* hybridisation or immunohistochemistry would demonstrate the true effect of these data. Moreover, many studies use multiple housekeeping genes in their analyses, so the addition of one or more to this data set could significantly improve the validity of control data.

In the view of aforementioned research, one possible interpretation of these findings is that the differences found in the behavioural phenotypes in *Igf2*-P0 KO mice are modulated by dysregulation of central 5-HT function, derived by a hyperactive HPA axis. This dysfunction of the 5-HT system might in turn act to; a) increase stress responses and anxiety among the *Igf2*-P0 KO mice by down-regulating $GABA_{\alpha}$ receptor expression, and, in parallel b) affect facets of working memory function (i.e. attentional function and impulse control) via altering prefrontal and striatal dopamine release. In order to provide stronger support for this proposal, a further expression analysis of genes involved in dopamine function (e.g. dopamine receptors D1 and D2 etc.), would be required. Additional work of fundamental importance, would be to demonstrate that the differences in GABA and 5-HT systems observed in the *Igf2*-P0 KO mice are absent in *Igf2*-Null KO mice, in order to ensure that these changes in receptor-gene expression underlie the behavioural differences among the *Igf2*-KO mice, rather than being non-related alterations in neural substrates as a results of early growth retardation.

Possible future work might also include pharmacological manipulations of GABA- and 5-HTergic systems in *Igf2*-P0 KO mice to try and normalise behaviour. Such experiment would show conclusively whether it is alterations in these systems that are giving rise to the aberrant behavioural phenotypes in your mutant mice.

7.4.1 Summary of key results from Chapter VII

- A significant reduction was found in hippocampal expression of the $GABA_{\alpha 3}$ receptor subunit in the *Igf2*-P0 KO mice when compared to their WT littermates, however no difference was observed in $GABA_{\alpha 2}$ receptor subunit expression or in $GABA_{\alpha 3}$ receptor subunit expression in the hypothalamus.
- In the Igf2-P0 KO mice, there was a significant reduction in the expression of $5\text{-}HT_{2A}$ receptor subtype in the hippocampus relative to their WT littermates, although no difference was found in hippocampal $5\text{-}HT_{1A}$ receptor expression.
- There was no difference found in the expression of $5\text{-}HT_{2A}$ or $5\text{-}HT_{2C}$ receptor subtypes in the striatum, between Igf2-P0 KO mice and their WT littermates.
- A significantly greater splicing of the non-truncated functional variant of the $5\text{-}HT_{2C}$ receptor was found in the striatal tissue of the *Igf2*-P0 KO mice when compared to that of their own WT littermates

Chapter VIII

General Discussion

Nutrition is the major intrauterine environmental influence that alters expression of the foetal genome and can have deleterious and lasting consequences for mental health in later life. In fact, there is evidence that the intrauterine environment of the individual foetus may be of at least equal importance in the aetiology of chronic disease in adults as the genome of the foetus (Wu et al., 2004). The scope of the present thesis was to investigate how compromised nutrient availability, via placental deficiency and growth retardation in intrauterine life, might act upon brain development and behavioural phenotypes in later life. This was achieved by comparing performance of two different *Igf2* imprinted gene knockout (KO) models (i.e. the *Igf2*-P0 KO and *Igf2*-Null KO lines) to wild-type (WT) littermate controls, on a wide range of behavioural tests. Furthermore, a preliminary examination of changes in gene expression was also carried out, to dissociate some of the conceivable neural substrates that may underlie the observed behavioural phenotypes.

8.1 Main findings

The generation of the growth deficient *Igf2* KO mouse models was successful with relatively low neonatal mortality. Mice of both *Igf2*-P0 and *Igf2*-Null KO lines showed considerable intrauterine growth retardation (IUGR) as indicated by the lowered birth weight, although this growth deficiency was found to be more severe in *Igf2*-Null KO mice. The weight of *Igf2*-P0 KO mice was ~75% that of WT controls at birth, but these mice showed a considerable compensatory weight gain during early postnatal life and were found to have equivalent body weight to their own littermate-controls (i.e. WT-P0 controls) in adulthood (Chapter III). In addition, early somatic development did not seem to be affected in *Igf2*-P0 KO mice. In comparison, the birth weight of the *Igf2*-Null KO mice was only ~55% of their WT-littermate weight. Moreover, while the *Igf2*-Null KO mice showed a transient growth spurt in the first few days following birth, these mice were delayed on all measurements of somatic

development (i.e. unfolding of ear-pinna, incisor eruption, fur growth and eye opening) and remained growth impaired throughout life.

8.1.1 Behavioural findings

In general, the *Igf2*-P0 KO mice selectively showed a marked increase in stress reactivity and anxiety-related behaviour, across a number of ethologically based behavioural batteries that are sensitive for traits of anxiety in rodents (Chapter IV and see Table 8.1). The *Igf2*-P0 KO mice displayed a heightened startle response to acoustic stimuli, greater neophobic reactivity to a novel food, spent proportionally less time on the open arms of the elevated plus-maze (EPM) and showed a greater latency to ambulate into the exposed open region of the open field (OF) arena. Moreover, *Igf2*-P0 KO mice spent less time in the novel compartment and were more reluctant to enter the distal part of the novel compartment, in the free exploratory paradigm (FEB). The presumption that these changes in behaviour reflected heightened stress and anxiety was further validated by the fact that the *Igf2*-P0 KO mice were found to be indistinguishable from WT controls on every parameter of locomotor competence when assessed in these paradigms (i.e. ambulatory velocity, duration of ambulation and distance ambulated). Furthermore, no differences in motor ability were found between the *Igf2*-P0 KO mice and WT controls when assessed in the locomotor activity (LMA) chambers or on the rotarod apparatus. Conversely, *Igf2*-Null KO mice did not display any evidence of increased stress or anxiety phenotypes, but instead showed a significant reduction in ambulation during the LMA chambers, as well as an overall improvement in motor co-ordination and balance on the rotarod apparatus (indicated by greater latency to fall from the rod and a greater rotation velocity at the time of fall). Although the improvements in rotarod performance of the *Igf2*-Null KO mice could readily be accounted by their relatively smaller body size, the reasons behind the reduction in LMA is less clear. In terms of sensory-motor gating, the *Igf2*-P0 KO mice displayed a mild impairment in prepulse inhibition (PPI), relative to WT-control mice; however *Igf2*-Null KO mice did not differ from WT controls on this measure.

Table 8.1: *Main findings of the behavioural differences observed among the Igf2-KO mouse models (relative to WT-controls), in the present research.*

Behavioural test:	Parameter/construct	Igf2-P0 KO	Igf2-Null KO
LMA chambers	Ambulation	No difference	**Lowered**
Rotarod apparatus	Motor balance and coordination	No difference	**Heightened**
Accoustic startle and PPI	Stress response	**Heightened**	No difference
	Sensory motor gating	**Lowered**	No difference
EPM	Anxiety-related behaviour	**Heightened**	No difference
OF	Anxiety-related behaviour	**Heightened**	No difference
FEP	Anxiety-related behaviour	**Heightened**	No difference
5-CSRTT	Attention	**Heightened**	**Heightened**
	Impulse control	**Heightened**	No difference
	Motivation	No difference	**Lowered**
SSRTT	Go responses	No difference	No difference
	Stop responses	No difference	No difference

Heightened attentional performance was observed for both *Igf2*-P0 and *Igf2*-Null KO mice during the 5-CSRTT relative to WT controls, as indexed by greater discriminative response accuracy (Chapter V). This improvement in response accuracy was observed in *Igf2*-P0 KO mice during task acquisition, at baseline, and during task-manipulations which increased attentional demand (i.e. at increased ITI and white-noise distraction). Similarly, the response accuracy of *Igf2*-Null KO mice was noted to be significantly improved at baseline, during sessions where the temporal predictability of the stimuli was absent and under specific task-manipulations during the 1-choice serial reaction time task (1-CSRTT, i.e. at increased ITI, and white noise distraction, data not shown). Moreover, *Igf2*-P0 KO mice showed greater impulse control during the 5-CSRTT, indexed by reduced numbers of premature nose-pokes, relative to WT controls, both at baseline and in sessions when the ITI duration was pseudorandomly extended. As expected, the number of trials initiated during the 5-CSRTT was considerably reduced for *Igf2*-Null KO mice as these mice are likely to become sated much earlier during each session relative to *Igf2*-P0 KO and WT controls, as a result of their lower body weights (and size). Importantly, since no differences were found between the *Igf2*-P0 KO mice and the WT controls in the Stop signal serial reaction time task (SSRTT, Chapter VI), it is

feasible that increased behavioural inhibition among the *Igf2*-P0 KO mice stems from aspects of cognitive inhibition rather motoric inhibition.

8.1.2 Consistency of behavioural findings across different WT-control lines.

In addition to the behavioural differences that were demonstrated between the IUGR *Igf2*-KO mouse models and the WT-control group, some notable variations were also found between the WT littermates of the *Igf2*-P0 and *Igf2*-Null mutant lines (i.e. the WT-P0 and WT-Null controls). The greatest differences were observed on parameters of somatic developmental and postnatal growth; however several variations were also noted in behavioural measures which could be accounted for by the increased body size and mass of the *Igf2*-Null derived WT controls. Thus, the heightened startle response of the WT-P0 controls where body mass may influence the sensitivity of the startle measurement, and the improved rotarod performance due to lower body/rod size ratio among WT-P0 controls, may be explained in terms of different body size/weight. However, other differences, such as the increased activity level of WT-Null controls during the EPM and the increased number of nose-pokes and panel-pushes during the early acquisition of the SSRTT could not be attributed solely to body size/weight differences, and suggest that some other factors may contribute to these differences. Nevertheless, it is highly unlikely that the variations between the two WT-control groups has had much of an effect on interpretation of the observed behavioural differences between the *Igf2*-P0 KO and *Igf2*-Null KO mice, and the collective WT-control group, since no difference was ever found on any of the relevant behavioural parameters between the segregated WT-P0 and WT-Null controls.

Furthermore, in order to further ensure that the main behavioural differences observed between the *Igf2*-P0 KO mice and the combined WT-control group was not produced (or exacerbated) by discrepancy in performance of either individual WT-control group, additional statistical comparisons were carried out between *Igf2*-P0 KO mice and each of the segregated WT-control groups on the main behavioural parameters (see appendices 8.1 and 8.2). In short, the heightened stress- and anxiety-related behaviour of *Igf2*-P0 KO mice remained significant when compared to both WT-P0 and WT-Null controls for the relevant parameters of the acoustic startle and PPI paradigm (i.e. the increased startle response), food neophobia assessment (i.e. the

lowered consumption of novel food-substance) and the OF test (i.e. the increased latency of first entry to central region). The lowered exploratory behaviour of *Igf2*-P0 KO mice on the open arms of the EPM was only found to be significant when compared to behaviour of their own WT-littermate controls, whereas the anxiogenic effects noted on the FEP parameters only yielded significance relative to WT-control mice of the *Igf2*-Null line. Similar patterns were found following separate comparison between the *Igf2*-KO mice and both WT-control groups with regard to the improved 5-CSRTT performance. Taken together, these findings provide further evidence that the main behavioural findings were not misconstrued due to heterogeneity between the WT-control groups of different mutant lines.

8.1.3 Molecular findings

A preliminary molecular analysis was carried out between the mice of the *Igf2*-P0 KO mice and their own respective WT-littermate controls, using real-time qPCR for specific gene candidates related to the main behavioural differences found (Chapter VII). Receptor subtype-specific alterations were found in gene expression for $GABA_{\alpha 3}$ and $5\text{-}HT_{2A}$ receptor subunits, suggestive of irregular GABA and 5-HT transmission function in the hippocampus. Furthermore, there was significantly greater splicing of the non-truncated functional variant of the $5\text{-}HT_{2C}$ receptor found in the striatal tissue of the *Igf2*-P0 KO mice when compared to that of their own WT littermates, evidence of altered post-transcriptional modification of the $5\text{-}HT_{2C}$ receptor. Unfortunately, an equivalent comparison of gene expression could not be carried out between KO mice and WT littermates of the *Igf2*-Null line, due to both temporal and financial constraints, but would be useful as these mice did not show anxiogenic behaviours but were IUGR. Therefore, such further examination would establish a stronger functional linkage between the observed alterations in gene expression and the behavioural phenotypes in *Igf2*-P0 KO mice.

8.2 Relationship between behavioural phenotypes

The co-occurrence of heightened stress/anxiety-related phenotypes in the *Igf2*-P0 KO mice, together with elevated attentional-function and impulse control as displayed on the 5-CSRTT, is particularly interesting given the fact that anxious individuals frequently tend to experience increased arousal, behavioural inhibition,

and heightened awareness of their surroundings (Enoch, White, Waheed and Goldman, 2008). Moreover, behavioural inhibition during early childhood, frequently highlighted by heightened withdrawal and vigilance towards novel people, objects and environment, has been implicated as a risk factor in the development of anxiety disorders (Hirschfeld-Becker, Micco, Henin, Bloomfield, Biederman and Rosenbaum, 2008; Pérez-Edgar and Fox, 2005; Gray and McNaughton, 2000). In fact, the physiology of behaviourally inhibited children has been shown to be paralleled by activation of the physiological systems that are affiliated with increased stress and anxiety (Fox, Henderson, Marshall, Nicholsand Ghera, 2005). For example, children showing high levels of behavioural inhibition show significant increases in heart rate and higher basal cortisol levels after presentation of a stressor (Schmidt et al., 1997; Kagan et al., 1988; Kagan et al., 1987). Nevertheless, the extent to which these symptoms of behavioural inhibition in humans are akin to the impulse control observed in rodents during the 5-CSRTT, would be a subject of debate.

Despite the large amount of research that has been dedicated to the area of selective attention (or attentional bias) towards aversive stimuli in cases of anxiety disorders (e.g. Cisler and Koster, 2010; Rinck, Becker, Kellerman and Toth, 2003; Mathews, Mackintosh and Fulcher, 1997), at present very few studies have more generally focused on the relationship between anxiety and attentional competence *per se* (Castaneda, Tuulio-Henriksson, Marttunen, Suvisaari and Lönnqvist, 2008). However, in a recent study individuals with a life long diagnosis of noncomorbid anxiety disorders were found to have significantly elevated auditory P300 potential (an event related voltage considered to index attentional allocation) and improved performance on the Wechsler Adult Intelligence Scale-Revised (WAIS-R, Wechsler, 1981) digit symbol subtest, when compared to either individuals of comorbid anxiety disorders (i.e. major depression and alcohol use disorder) or nonpsychiatric controls (Enoch, White, Waheed and Goldman, 2008). Also of considerable importance, a Val158Met polymorphism of the catechol-*O*-methyltransferase (COMT), a dopamine-degrading enzyme, has been implicated with patterns of cognitive function and emotionality similar to that observed in the *Igf2*-P0 KO mice (Enoch et al., 2008). In particular, individuals with the Met158 allele, which leads to reduced COMT activity, have been found to perform better on tests of working memory and executive function, (Enoch, Waheed, Harris, Albaugh and Goldman, 2009; Barnett, Jones, Robbins and Müller, 2007; Egan et al., 2001), but have a greater risk of developing

anxiety-related behaviours (Hetttema, et al., 2008; Olsson et al., 2005; Enoch, Xu, Ferro, Harris and Goldman, 2003). Consistent with this, COMT inhibitors have been found to improve working memory and attention (Apud et al., 2007; Tunbridge, Harrison and Weinberger, 2006). Based on these findings, it is possible that altered function of dopaminergic pathways might be among the key neural substrates that underlie the behavioural phenotypes *Igf2*-P0 KO mice.

8.3 Nutritional deficiency or intrauterine growth restriction?

The observation that only *Igf2*-P0 KO mice (and not the Igf2-Null KO mice) showed heightened stress reactivity, anxiety-related behaviour and greater impulse control, raises the question of whether these particular differences in behavioural phenotypes (see Table 8.1) might be particular sequelae of foetal nutritional deficiency, as opposed to a result of IUGR *per se*. The key difference between the *Igf2*-KO mouse models lies in the pattern of deletion of *Igf2*, where foetal expression was intact in *Igf2*-P0 KO mice but not *Igf2*-Null KO mice, whilst both *Igf2*-KO mice had no placental *Igf2* expression, and it has been proposed that a mismatch between foetal and placental *Igf2* expression will affect the relative demand and transmission of nutrition (see Angiolini et al., 2006 and Reik et al., 2003). Thus *Igf2*-P0 KO mice will have high demand (intact foetal *Igf2*) but a reduced placental capacity and may therefore have been more severely affected by nutritional deficiency than mice of the *Igf2*-Null KO model. In contrast, as the ablation of *Igf2* expression from the foetal tissue of *Igf2*-Null mice exerts limitations on the their intrinsic growth trajectory, it is plausible that the nutrient demand of the foetus was at least, to some extent, reduced in parallel with the reduced placental supply of vital nutrients; thus these mice were not as nutritionally constrained despite their IUGR.

Interestingly, recent data on the *Igf2*-P0 KO model illustrated an up-regulation of glucose and System A amino acid transport of the placenta at E16 (Coan, Angiolini, Sandovici, Burton, Constância and Fowden, 2008; Constância et. al., 2005). However, this up-regulation was decreased at E19 in correlation with reductions in foetal weight (relative to wild-type weight) and thus failed to rescue the foetus from IUGR, whereas such up-regulation of glucose and amino acids has previously been found to be absent in the placentas of *Igf2*-Null KO mice (Angiolini et al., 2006). In agreement with these studies, inverse correlations have been noted

between System A amino acid transporter activity and birth size and weight in babies across the range of normal birth weight and size (Godfrey et al., 1998). One interpretation of these findings is that a placental adaptation occurs in response to signals from foetal tissue still expressing *Igf2*, in terms of up-regulation of glucose and System A amino acid transport from the placenta, in effort to retain normal foetal growth (Constância et al., 2005). However, because of failure to maintain this up-regulation (along with the decreased placental permeability), foetal growth restriction ensues during late gestation. In comparison, a reduced (or absent) nutrient demand signal from the foetus of the *Igf2*-Null KO model would require smaller placental supply of nutrients to support the lower growth rate, and placental glucose and amino acid transport activity is therefore lowered accordingly.

However, since heightened attentional function was observed across both *Igf2*-KO mouse models, it is possible that the neuronal substrates that underlie this behavioural difference are either more susceptible to early nutritional deficiency than the heightened stress/anxiety and impulse control (assuming that the *Igf2*-Null KO mice were exposed to at least a mild malnutrition), or more likely to result from IUGR (at least to a greater degree) rather than nutritional deficiency.

8.4 Implications of early postnatal compensatory growth

One of the main developmental differences between the *Igf2*-KO mouse models was their distinct postnatal growth pattern (Chapter III) where mice of both *Igf2* KO models displayed some degree of accelerated postnatal weight gain during early life. However, only *Igf2*-P0 KO mice showed a complete compensatory (or 'catch-up') growth, as indicated by an equivalent body weight to their own WT littermates at day 100. In comparison, *Igf2*-Null mice showed only a brief growth spurt during the first few days post partum and remained growth retarded throughout life. Therefore, it could be argued that behavioural differences between *Igf2*-P0 and *Igf2*-Null mice might be due to the variation in early postnatal growth rather than differences in nutritional supply during intrauterine life. Such an interpretation could be considered in line with the findings of significantly greater body weight at day 100 of the WT littermates of the *Igf2*-Null mutant line (relative to littermates of the *Igf2*-P0 control line), suggesting that the *Igf2*-Null KO mice might possibly have gained less nutrient access to their mother during lactation. However, this is unlikely to have

been the case since litter size, in terms of the number of WT littermates, did not significantly affect the body weight (or size) of *Igf2*-Null KO mice at postnatal day 100. Thus it is more probable that the lasting growth impairment of *Igf2*-Null KO mice stems from a genetically intrinsic limitation in growth potential due to the ablation of foetal *Igf2* expression, rather than due to poor nutrient access in early postnatal life.

One of the prominent theoretical paradigms in the research of foetal programming, known as the 'thrifty phenotype hypothesis' (Hales and Ozanne, 2003), highlights the importance of the interaction between intrauterine and postnatal growth for later disease susceptibility. As such, the foetal endocrine system undergoes adaptive change in response to a poor nutrient environment, in an attempt to divert the limited nutrient supply to maintain survival and development of vital organs (e.g. the brain), at the expense of overall growth leading to reduced birth sizes of the organism (Cianfarani, Germani and Branca, 1999). Nevertheless, while this adaptive response might have significant survival advantages under conditions where nutrition is intermittent or poor, these same physical adaptations could become detrimental in a postnatal environment where nutrition is abundant. This hypothesis has gained considerable support from a number of epidemiological studies which suggest that individuals that have undergone IUGR but exhibited early postnatal compensatory weight gain ('catch-up growth') and have above average body weight (or size), are at greater risk for metabolic (Forsen et al., 2000; Ong et al., 2000) and cardiovascular diseases (Eriksson et al., 1999; Leon et al., 1996). However, many other studies have failed to replicate these findings (Martin, McCarthy, Smith, Davies and Ben-Schlomo, 2003; Law et al., 2002) and in terms of neurological or psychological disorders, there has not been any evidence indicating such effects on neurodevelopment or behaviour.

One of the major challenges to the thrifty phenotype hypothesis is in regard to the vague definition of 'catch-up' growth. This term usually refers to the realignment of the genetic growth potential of the individual and can refer to the first 6-12 months of life or to as late as two years after birth (Simmons, 2005). However, considering the fact that approximately 90% of IUGR infants display some catch-up growth within the first six months of life (McMillen and Robinson, 2005), it is reasonable to assume that this early postnatal weight gain might simply represent a normal physiological response to the foetal growth deficiency rather than being an indicator of an aberrant adaptation due to nutritional imbalance. As such, these studies are

compromised by an inability to dissociate the extent of *in utero* growth deficiency between children showing catch-up growth and those which do not. Therefore, it is possible that the early catch-up growth displayed by the *Igf2*-P0 KO mice might merely reflect a normal physiological response to suboptimal nutrition in early life and the *Igf2*-Null KO mice however, did not show catch-up growth as they were malnourished to a lesser degree *in utero*. Another critical shortcoming of such an interpretation of the thrifty phenotype hypothesis involves the lack of evidence that catch-up growth does in fact mirror nutritional imbalance rather than the influences of other environmental or non-environmental factors. For example, children of low birth weight which show postnatal catch-up growth are more likely to have been subjected to maternal smoking during foetal life (Ong et al., 2000). Perhaps more fundamentally, the thrifty phenotype hypothesis also does not address the possibility that the postnatal growth rate of these presumably catch-up and noncatch-up growth children might not reflect a qualitative difference in postnatal environment (such as varying nutrient availability), but merely constitute the opposite tails of the normal distribution of postnatal growth rate.

8.5 A role for dysregulated imprinted gene expression in foetal development and adult brain function

During gestation and early postnatal life in particular, the developmental trajectories of neurons are determined by a complex series of epigenetically-mediated processes. Furthermore, there has been increasing body of work implicating that environmental factors can act to alter these trajectories via modulating histone modifications and DNA methylation. In fact, the notion that epigenetic dysregulation of imprinted gene expression might play a paramount role in modulating the effects of early life adversities on adulthood disease susceptibility has gained substantial support among researchers (Lim and Ferguson-Smith, 2010; Dolinoy, Das, Weidmand and Jirtle, 2007; Waterland and Michels, 2007; Gallou-Kabani and Junien, 2005; Abel, 2004). As imprinted genes are functionally haploid and expression is tightly epigenetically regulated, they may be particularly susceptible to environmental/nutritional insults (Thompson, Konfortova, Gregory, Reik, Dean and Feil, 2001), for example by a lack of dietary methyl donors and cofactors owing to the lack of compensative protection that diploid expression usually provides (Jirtle and

Skinner, 2007; Waterland and Jirtle, 2004; Vand den Veyver, 2002). Moreover, as imprinted genes are important in fundamental foetal growth and development processes, any maternal diet-induced dysregulation of imprinted gene expression in the embryo/foetus, may have severe adverse consequences for foetal development. In agreement with this, *in vitro* studies have described altered expression of the imprinted *H19* and *Igf2* genes following modification of the external nutrient supply (Doherty, Mann, Tremblay, Bartolomei and Schultz, 2000; Khosla, Dean, Reik, and Feil, 2001), whilst exposure to a nutrient-restricted diet during early postnatal life was associated with developmental relaxation of *Igf2* imprinting in mice (Waterland et al., 2006). Furthermore, in a recent study of the Dutch Hunger Winter cohort, subjects that were exposed to the famine during gestation were found to have a persistent and significant reduction in methylation at the *IGF2* locus (Heijmans et al., 2008). However, the extent to which these epigenetic changes may confer vulnerability to later mental illness remains to be empirically tested.

Besides affecting imprinted gene expression in the foetus directly, nutritional and other environmental insults also influence foetal development indirectly via effects on imprinted genes expressed in the placenta. Many placentally expressed imprinted genes identified to date are known to be involved in regulating and allocating maternal nutrient resources to the foetus; hence any perturbation of their function could potentially impair placental efficiency and reduce the supply of nutrients (possibly via amino acid transport system A) and oxygen to key organs, including the embryonic brain. Several human studies have described altered placental expression of imprinted genes in IUGR or small for gestational age (SGA) pregnancies, including enhanced expression of the maternally expressed gene *PHLDA2* and suppression of the *IGF2* (Apostolidou et al, 2007; Diplas et al, 2009; McMinn et al, 2006). Consistent with this finding, a recent study employing genome-wide methylation analysis has revealed a significant hypomethylation of the *H19/IGF2* imprinting control region, in IUGR placentas (Bourque, Avila, Penaherrera, von Dadelszen and Robinson, 2010).

8.6 The advantages and limitations of animal models

A range of different animal species have been used as models of intrauterine nutritional deficiency and foetal growth restriction as the majority of the important

experimental research cannot be adequately undertaken in humans for both ethical and practical reasons. Moreover, because the mammalian systems of rodents share embryological, anatomical and physiological homology with humans, studies using rodent models have provided valuable translational insights into the genetic, molecular and cellular events that underlie the foetal programming of later health vulnerability in humans (Vuguin, 2007). However, caution must be exercised when making inferences about the developmental implications of early life adversities from animal models to human conditions. One of the major caveats of using animal models to emulate foetal programming in humans is the fact that the ontogeny of central nervous system (CNS) in rodents deviates markedly from humans such that the critical periods for neural development in rodents extends into early postnatal life, whereas the majority of the maturation of the human brain has largely taken place by the time of birth (Rice and Baron Jr., 2000), although there are regionally specific trajectories in the development of some brain structures that are not complete by adulthood (e.g. prefrontal cortex, see Diamond, 2002). Nevertheless, despite the fact that animal models will probably never be totally satisfactory in mimicking the condition and consequences of human pregnancy and development these models have proven to be effective and valuable tools to further our understanding of the basic mechanisms underlying foetal growth and programming and are necessary for the advancement of this field of research.

8.6.1 The advantages and limitations of the Igf2-KO models

The application of the *Igf2* KO mouse lines provides an effective approach to model and investigate the relationship between foetal life and later health. The targeted deletions of the imprinted *Igf2* gene in these models are particularly appropriate to assay nutritional programming given the fact that the foetal IGF axis has been shown to be regulated by nutrition (see Chapter I, section 1.4.1.2), and also in light of accumulating evidence for the role of placentally expressed imprinted gene in IUGR in humans (see section 8.7). While established rodent models have in general been considered to be beneficial for developmental research due to their short generation time and life span and the ability to produce large litters (Carter, 2007), the *Igf2*-KO mouse models extend several other advantages over previous animal models of early life nutrition. For instance, the fact that the *Igf2*-P0 and Null deletions inhibit

the genetic growth potential of the placenta intrinsically, no imposition of food deprivation or malnutrition is required and thus maternal distress is minimized among the dams, reducing potentially confounding maternal effects on postnatal development and behaviour (see Chapter I, section 1.5.2). Also importantly, since the *Igf2*-P0 and Null mutations are only passed down to ~50% of offspring these genetic mouse models allows the foetally growth deficient *Igf2*-KO mice to be directly compared with their own WT littermates; therefore ensuring greater homogeneity of the postnatal environment between experimental and control groups.

However, there are some issues in the use of the *Igf2*-KO models that require discussion. For example, while compromised placental efficiency will give rise to a reduction in placental transport of nutrients to the foetus (i.e. glucose, lactate and amino acids), other critical functions of the placenta are likely to be affected as well, such as oxygen delivery and regulation of hormonal balance (see Barry, Rozance and Anthony, 2008 and McMillen and Robinson, 2005 for review). Therefore, the possibility that, for example, either foetal hypoxia and/or over exposure of glucocorticoids, could have played contributing roles to the brain and behavioural phenotypes of the *Igf2*-KO mice in adult life cannot be dismissed. In fact, KO mice deficient for the placentally-expressed 11β-hydroxysteroid dehydrogenase type 2 gene (11β-HSD2-Null), a catalyst of glucocorticoid synthesis, have been found to be foetally growth retarded and exhibit heightened anxiety in later life (Holmes et al., 2006). Evidently, these phenotypic similarities between the 11β-HSD2-Null mice and the *Igf2*-P0 KO mice highlight the developmental susceptibility of the stress/anxiety neural circuitry to foetal programming. However, on the basis that several studies have indicated placental deficiency in cases of foetal growth deficiency in humans (see section 8.5 above), it is reasonable to assume that changes in oxygen delivery and hormonal exposure may be inevitable physiological consequences of malnutrition in early life.

Furthermore, while animal models such as the *Igf2*-KO lines provide a relatively high degree of experimental control over confounding variables, it is difficult, if not impossible, to exclude subtle early postnatal life confounds such as; differential mother-pup or pup-pup interactions, as results of any of the physiological or behavioural variations across genotypes, but these should be reduced in comparison to models of maternal malnourishment. Another general disadvantage of using a mouse model for a functional study is the relatively small size of mice (Carter, 2007),

which severely affects the quality of spatial resolution in any morphological or neurochemical assay. However, this limitation is being addressed by the ongoing development of sophisticated techniques for neuronal and molecular imaging.

8.7 Implications of present work and future directions

This thesis introduced, for the first time, a genetic model to investigate the lasting impact of adverse nutritional environment upon brain development and behaviour in later life. The experiments reported here provide converging lines of evidence, both behavioural and molecular, that are consistent with the view that nutrient deficiency arising from compromised placental function affects expression of the foetal genome, which in turn leads to lasting changes in both brain and behavioural phenotypes. Furthermore, although there has been significant discussion on the possible role of imprinted genes in mediating the relationship between the foetal environment and later mental health (Kopsida, Mikaelsson and Davies, 2010; Lim and Ferguson-Smith, 2009; Dolinoy et al, 2007; Gallou-Kabani and Junien, 2005; Abel, 2004), the present research is the first to provide direct evidence for this link. The behavioural findings of heightened stress and anxiety in the *Igf2*-P0 KO mice substantiate earlier work from humans and animals on early life nutrition (see Chapter IV), and is also in accordance with work describing increased HPA activity in cases of IUGR (see Chapter VI, section 4.4.). However the evidence suggesting improved executive function (i.e. better discriminative response accuracy and greater impulse control during the 5-CSRTT) is entirely novel and are particularly intriguing given the previous epidemiological studies indicating a higher risk of attention-deficit hyperactivity disorder (ADHD) in children born IUGR (see Breslau et al., 1996 for review). These findings could suggest that other environmental factors (besides poor nutrition) which are known to affect foetal growth might underlie the relationship between IUGR and ADHD risk (see Chapter V, section 5.1). However, it could also be argued these findings highlight the susceptibility of the neurobiological substrates of attention and behavioural inhibition to foetal programming by early nutritional deficiency, and that the differential behavioural manifestations of this compromised neural circuitry reflects between-species variation.

The work in this thesis provides only a snap-shot view of the mechanisms and consequences of nutritional programming, and raises a number of important questions

that need to be addressed with further research. For example, while *Igf2*-P0 KO mice repeatedly displayed evidence of heightened anxiety towards a potentially aversive environment during the approach-avoidance conflict tests, it remains unknown how *Igf2*-P0 KO mice would react to the presentation of an aversive stimulus (i.e. fear reactivity). Although the differences between the behavioural responses to a potential (i.e. anxiety) and a present threat (i.e. fear) may appear to be only subtle, there is some evidence that these behavioural constructs rely on the involvement of distinct brain regions (Barkus et al., 2010; Bannerman et al., 2004). As such, the hippocampus has been more strongly implicated in anxiety-related behaviours such as observed during ethological conflict tests (i.e. context based response), whereas fear reactivity during a fear conditioning paradigm (i.e. cue based response) has been shown to rely to a greater degree the amygdala (see Bannerman et al., 2004 for review). Therefore, a further assessment of fear expression and learning during a fear conditioning paradigm (see Ehrlich et al., 2009), would provide a greater clarity of the scope of the heightened emotionality of *Igf2*-P0 KO mice, and dissociate the roles of the hippocampus and the amygdala, in these stress- and anxiety phenotypes. To further corroborate this dissociation, gene expression analysis of key anxiety-related targets in the amygdala could also be investigated in *Igf2*-P0 KO and WT-littermate mice (see Chapter VII).

Furthermore, in light of the large amount of research that has highlighted the developmental susceptibility of the hippocampus (see Chapter I, section 1.3) and the indications of altered neurotransmitter functionality in this brain region of *Igf2*-KO mice (Chapter VII), memory function should be also be assessed, as it has been shown to depend heavily on the integrity of the hippocampus (e.g. Bannerman et al., 2004). Therefore further behavioural characterization of the *Igf2*-KO mice should include assessments of this cognitive ability. Such an investigation would also be of particular relevance, considering the fact that epidemiological studies have indicated that IUGR children are at higher risk of memory impairments in later life (Geva, Eshel, Leitner, Fattal-Valevski and Harel, 2006; Vicari et al., 2004). However, the experimental design should minimize the involvement of high levels of visuospatial processing (in light of the heightened attentional performance of *Igf2*-P0 KO mice) and a test such as the Social Transfer of Food Preference (STFP, see Wrenn, Harris, Saavedra, and Crawley, 2003) should be used, in which memory function is examined via olfactory functioning of subjects.

Another important line of future research, would be to follow-up on the findings of the improved attentional function and impulse control during the 5-CSRTT (Chapter V). For instance, it would be interesting to examine *Igf2*-P0 KO mice on tasks of cognitive flexibility (i.e. the ability to spontaneously withhold, modify or sustain adaptive behaviour in response to changing situational demand), since findings from recent work by Robbins and colleagues (2008) have raised the possibility of a functional dissociation between impulse control and cognitive flexibility. In previous work, systemic striatal administration of the specific 5-HT_{2C} receptor antagonist SB242084, has been shown to increase impulsive behaviour of mice on the 5-CSRTT, however in a subsequent study, the administration of SB242084 was also found to improve spatial reversal learning in mice (Boulougouris, Glennon and Robbins, 2008). The reverse effects were observed following administration of M100907, a specific 5-HT_{2A} antagonist (Boulougouris et al., 2008). Hence, in context of the evidence of altered 5-HT_{2C} receptor splicing in *Igf2*-P0 KO mice (Chapter VII), it is highly relevant that an assessment of cognitive flexibility is performed, using a number of the tasks that have been developed to assay such frontal cortical functions, such as the attentional set shifting paradigm (formally equivalent to the human Wisconsin card sorting test, Colacicco et al., 2002) and serial spatial reversal learning (Boulougouis et al., 2008).

The preliminary work presented here revealed evidence of altered GABA and 5-HT function (i.e. reduced $GABA_{\alpha 3}$ and 5-HT_{2A} receptor expression) in *Igf2*-P0 KO mice as result of compromised nutrient access and growth in foetal life. However, while the differences in the GABA- and 5-HTergic receptor expression of *Igf2*-P0 KO mice were predominantly in line with their behavioural phenotypes, it is difficult to conclude a functional linkage between these behavioural and molecular aspects without further research. As mentioned previously (Chapter VII), it is of fundamental importance to establish that the differences in gene expression in *Igf2*-P0 KO mice are absent in mice of the *Igf2*-Null KO line, as these molecular changes could relate to IUGR without having any modulating involvement for the behavioural phenotypes of *Igf2*-P0 KO mice. It is also apparent, that further evidence of altered GABA and 5-HT function is required as these changes in receptor expression could have occurred as an adaptive intracellular response, to counter extracellular neurochemical changes of opposing effects. As such, the reduced expression $GABA_{\alpha 3}$ and/or 5-HT_{2A} receptors could have been post-synaptic responses to excessive pre-synaptic GABA and/or 5-

HT release. In order to address this issue, further research could use *in vivo* microdialysis to obtain indices of GABA and 5-HT flux in specific brain regions (e.g. hippocampus, striatum and prefrontal cortex). Another important area for future research would entail providing further support for the involvement GABA and 5-HT receptors in the behavioural phenotypes displayed by *Igf2*-P0 KO mice by examining whether the behavioural differences observed between *Igf2*-P0 KO mice and their respective WT littermates could be reduced or eliminated, with the use of the relevant pharmacological manipulations. Such work could involve local (hippocampal) administration of benzodiazepine drugs or 5-HT_{2A} agonists prior to testing on the ethological tests of anxiety, or administration of a specific 5-HT_{2C} antagonist (e.g. SB242084) during the 5-CSRTT.

Based on the current findings and previous research, it was hypothesized that the early nutritional insult which the *Igf2*-P0 KO mice were exposed to (due to the placental deficiency), might have caused hyperactivation of the HPA-axis. This, in turn led to a dysregulation of the central 5-HT system (leading to greater impulse control) and induced heightened stress/anxiety through hippocampal GABA circuitry, and altered attentional function via the frontal dopaminergic (and or noradrenergic) pathways. However, although this conjecture may be reasonable in light of the evidence reviewed in this thesis, there is a great deal of further research required to provide more solid support for this notion. For instance, while altered HPA function has been consistently reported in different animal models of early malnutrition and growth retardation, such endocrine changes need to be established in the *Igf2*-P0 KO model (and the *Igf2*-Null KO model), by either measuring basal levels of glucocorticoids (as well as ACTH, or CRH levels), or by examining glucocorticoid/mineralocorticoid receptor expression in the hippocampus. Furthermore, it may be interesting to investigate the potential involvement of dopamine in mediating the altered attentional function of the *Igf2*-KO mice, by using either *in vivo* microdialysis to assess dopamine transmission in the forebrain, or real-time qPCR to examine expression level in the prefrontal cortex of genes involved in either function, such as the dopamine receptors *D1* and *D2*, the *TH* gene (encodes the enzyme tyrosine hydroxylase, the rate-limiting enzyme in dopamine biosynthesis), the dopamine transporter gene (DAT) and the catechol-*O*-methyl transferase (COMT, a dopamine degrading enzyme) gene.

The use of *Igf2* deletion models was particularly relevant for the purposes of this research (in light of the central role for the IGF axis in mediating developmental programming), there are a number of other placental gene deletion models that could be of utility in examining the long-term effects of compromised nutrient transfer in intrauterine life, such as the *Peg1*, *Peg3* and *Slc38a4* gene deletion models. Alternatively, genetic models using over expression of growth inhibiting imprinted genes could also be adopted for this purpose. For example, over-expression of the imprinted *Phlda2* gene (due to loss of imprinting), results in foetal and placental growth retardation and, similar to *Igf2*-P0 KO mice, low birth weight and an accelerated postnatal weight gain (Salas et al., 2004). Finally, since environmental factors can alter the epigenome, their ability to influence disease risk might involve epigenetic transgenerational inheritance (Jirtle and Skinner, 2007). Studies in rodents have demonstrated that nutritional deficiency during gestation could affect the incidence of diabetes and growth defects in second (F2) generation animals (Zambrano et al., 2005; Cesani et al., 2003). Hence, it would be particularly interesting to investigate whether the heightened stress/anxiety and impulse inhibition phenotypes observed in *Igf2*-P0 KO mice (presumably as a result of compromised placental function), could be propagated to the their own offspring (i.e. F2 generation). Such work could be carried out by comparing the behaviour of WT-offspring of the *Igf2*-P0 KO mice, to that of WT-offspring of the WT-littermates of the *Igf2*-P0 mutant line, in some or all the behavioural tests used in this thesis. Finally, it would also be of interest to investigate the long-term effects *Igf2* gene manipulation in female mice, as marked gender-specific differences have been noted in the development of the CNS (see de Vrie and Södersten, 2009 for review), and the work in this thesis made use of only male subjects.

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Appendices:

Appendix I

Appendix 1.1: *The results from an independent T-test analysis of early postnatal body weight and growth rate, between the WT-control lines (i.e. WT-P0 and WT-Null). *Significant at $p < 0.05$.*

Parameters of early postnatal growth	**Independent T-test:**
Weight at birth	$t(75)= -1.95$, n.s.
Weight at postnatal day 100	***$t(75)= -5.27$, $p= 0.001$**
Postnatal growth rate: Day 1-25	***$t(76)= -3.75$, $p= 0.001$**
Postnatal growth rate: Day 25-50	***$t(76)= 4.02$, $p= 0.001$**
Postnatal growth rate: Day 50-75	$t(76)= -0.77$, n.s.
Postnatal growth rate: Day 75-100	$t(76)= -0.48$, n.s.
Overall postnatal growth rate: Day 1-100	$t(76)= -1.06$, n.s.

Appendix 1.2: *The results from an independent T-test analysis of somatic/ neurological development during early postnatal life, between the WT-control lines (i.e. WT-P0 and WT-Null). *Significant at $p < 0.05$.*

Parameters of somatic/ neurological development	**Independent T-test:**
Ear opening	***$t(75)= -2.26$, $p= 0.05$**
Incisor eruption	***$t(75)= -3.07$, $p= 0.005$**
Fur growth	$t(75)= 0.00$, n.s.
Eye opening	$t(75)= -1.26$, n.s.

Appendix 1.3: *The results from an independent T-test and repeated measures analysis of performance on the locomotor activiy chambers, between the WT-control lines (i.e. WT-P0 and WT-Null).*

Parameters of somatic/ neurological development	**Independent T-test:**	
Beam-breaks at Session 1	$t(30)= 1.04$, n.s.	
Beam-breaks at Session 2	$t(30)= 0.69$, n.s.	
Beam-breaks at Session 3	$t(30)= 0.69$, n.s.	
Runs at Session 1	$t(30)= 0.19$, n.s.	
Runs at Session 2	$t(30)= 0.33$, n.s.	
Runs at Session 3	$t(30)= 0.00$, n.s.	
	Between subjects test:	**Interaction:**
Beam-breaks across sessions	$F_{1,30}= 1.03$, n.s.	$F_{2,60}= 0.07$, n.s.
Run across sessions	$F_{1,30}= 0.01$, n.s.	$F_{2,60}= 0.08$, n.s.

Appendix 1.4: *The results from repeated measures and independent T-test analysis of performance on the rotarod apparatus, between the WT-control lines (i.e. WT-P0 and WT-Null). *Significant at $p < 0.05$.*

Parameters of balance and motor control	**Between subject test:**	**Interaction:**
Latency to fall of rod	$F_{1,35}$= 2.27, n.s.	***$F_{3,113}$= 2.97, p= 0.05**
Velocity level at time of fall	$F_{1,35}$= 2.22, n.s.	***$F_{4,140}$= 2.61, p= 0.05**
	Independent T-test:	
Latency to fall of rod at 35rpm	***$t(35)$= 2.80, p= 0.01**	
Latency to fall of rod at 45rpm	***$t(35)$= 2.38, p= 0.05**	

Appendix 1.5: *The results from an independent T-test and repeated measures analysis of somatic/neurological development during early postnatal life, between the WT-control lines (i.e. WT-P0 and WT-Null). *Significant at $p < 0.05$.*

Startle response and prepulse inhibition parameters	**Independent T-test:**	
Startle response to acoustic stimulus	***$t(46)$= -2.02, p= 0.05**	
Performance across trials of varying prepulse sound levels	**Between subject test:**	**Interaction:**
Startle response	$F_{1,46}$= 3. 73, n.s.	$F_{2,77}$= 2. 15, n.s.
Prepulse inhibition	$F_{1,46}$= 1. 34, n.s.	$F_{3,138}$= 0.67, n.s.

Appendix 1.6: *The results from an independent T-test and repeated measures analysis of condensed milk preference and total volume liquid consumption, between the WT-control lines (i.e. WT-P0 and WT-Null).*

Paremeters	**Between subject test:**	**Interaction:**
Total liquid consumption across sessions	$F_{1,65}$= 0. 44, n.s.	$F_{4,279}$= 1.36, n.s.
Condensed milk preference at:	**Independent T-test:**	
Session 3	$t(65)$= -0.55, n.s.	
Session 4	$t(65)$= -0.61, n.s.	
Session 5	$t(65)$= -1.85, n.s.	
Session 6	$t(65)$= -0.96, n.s.	

Appendix II

Appendix 2.1: *The results from independent T-test of behavioural measures on the elevated plus-maze, between WT-P0 controls and WT-Null controls. While no difference was noted across the WT-controls of the two Igf2 knock-out lines on the anxiety-related measures, the WT-controls of the Igf2-Null line showed elevated levels of locomotor activity (i.e. total distance moved, amount time spent moving and average velocity of loc-motion, during the session).* $^{\Psi}$*Relative to time on both open and enclosed arms. *Significant at* $p < 0.05$.

Behavioural Measures	Independent T-test: WT-P0 controls/WT-Null controls
Percentage of time spent on open arms $^{\Psi}$	$t(42)= -1.31$, n.s.
Frequency of open arm entries	$t(42)= 1.78$, n.s.
Latency of first entry onto an open arm	$t(42)= 0.64$, n.s.
Rearing	$t(42)= 1.82$, n.s.
Head dips	$t(42)= 0.13$, n.s.
Stretch attends	$t(42)= -0.48$, n.s.
Total distance moved	***$t(42)= -3.31$, $p= 0.005$**
Amount of time spent moving	***$t(42)= -3.35$, $p= 0.005$**
Average velocity	***$t(42)= -3.12$, $p= 0.005$**

Appendix 2.2: *The results from independent T-test analysis of parameters of anxiety-related and locomotor aspects on the open field test, between WT-P0 controls and WT-Null controls*

Behavioural Measures	Independent T-test: WT-P0 controls/WT-Null controls
Percentage of time spent in the inner-square	$t(40)=-1.29$, n.s.
Percentage of time spent in the middle-square	$t(40)=-0.43$, n.s.
Frequency of inner-square entries	$t(40)=-0.07$, n.s.
Frequency of middle-square entries	$t(40)=-1.31$, n.s.
Latency of first entry into the inner square	$t(40)= 0.40$, n.s.
Total distance moved	$t(40)= 0.41$, n.s.
Amount of time spent moving	$t(40)= 1.56$ n.s.
Average velocity	$t(40)= 1.41$, n.s.

Appendix 2.3: *The results from independent T-test analysis of parameters of anxiety-related and locomotor aspects on the free-exploratory paradigm, between WT-P0 controls and WT-Null controls*

Behavioural Measures	Independent T-test: WT-P0 controls/WT-Null controls
Percentage of time spent in the novel compartment	$t(38)= 1.20$, n.s.
Percentage of time spent in the distal part of the novel compartment	$t(38)= -1.46$, n.s.
Frequency of entries into the novel compartment	$t(38)= 1.80$, n.s.
Frequency of entries into the distal part of the novel compartment	$t(38)= 0.03$, n.s.
Latency of first entry into the novel compartment	$t(38)= 0.19$, n.s.
Latency of first entry into the distal part of novel compartment	$t(38)= - 0.42$, n.s.
Total distance moved	$t(38)= 0.67$, n.s.
Amount of time spent moving	$t(38)= 0.56$, n.s.
Average velocity	$t(38)= -0.60$, n.s.

Appendix III

Appendix 3.1: *The results of repeated-measures analysis of condensed milk reinforcer preference and total volume consumption between WT-P0 controls and WT-Null controls, on the final day of habituation prior to 5-CSRTT.*

Statistical analysis	Test of between subject: GENOTYPE
Condensed milk preference	$t(35)= 0.89$, n.s.
Total volume consumption	$t(35)= 1.05$, n.s.

Appendix 3.2: *The results from repeated-measures analysis of performance of main behavioural measures at sessions of different SD criterion levels during 5-CSRTT acquisition, between WT-controls and WT-Null controls.*

Behavioural Measures	Repeated-measures analysis of 5-CSRTT acquisition	
	Test of between-subject: GENOTYPE	Test of within-subject: INTERACTION
Trials	$F_{1,35}= 0.00$, n.s.	$F_{6, 214}= 0.87$, n.s.
Accuracy	$F_{1,35}= 0.36$, n.s.	$F_{10, 350}= 1.26$, n.s.
Omissions	$F_{1,35}= 2.28$, n.s.	$F_{4, 152}= 1.42$, n.s.
Correct RT	$F_{1,35}= 1.39$, n.s.	$F_{2, 52}= 0.96$, n.s.

Appendix 3.3: *The results from independent T-test of behavioural measures at 5-CSRTT baseline level, between WT-P0 controls and WT-Null controls.*

Behavioural Measures	Independent T-test: WT-P0 controls/WT-Null controls
Trials	$t(35)=-0.74$, n.s.
Accuracy (%)	$t(35)=- 1.35$, n.s.
Omissions (%)	$t(35)=- 0.12$, n.s.
Correct reaction time (sec)	$t(35)= 0.01$, n.s.
Magazine latency	$H_1= 3.18$, n.s.
Eating duration	$t(35)= .00$, n.s.
Premature nose-pokes	$t(35)=- 0.23$, n.s.
Nose-pokes per trial	$t(35)= 0.51$, n.s.
Panel-pushes per trial	$t(35)= 1.55$, n.s.

Appendix 3.4: *The results from a repeated-measure analysis of response patterns in discriminative response accuracy, rates of omissions and correct RT, between separate WT-control lines, WT-P0 controls and WT-Null.*

Behavioural Measures	Spatial location of light stimulus presentation	
	Test of between-subject: WT CONTROL	Test of within-subject: INTERACTION
Accuracy	$F_{1, 35}$= 0.02, n.s.	$F_{3, 106}$= 2.03, n.s.
Omissions	$F_{1, 35}$= 0.53, n.s.	$F_{4, 140}$= 2.21, n.s.
Correct RT	$F_{1, 35}$= 0.03, n.s.	$F_{2, 77}$= 0.15, n.s.

Appendix 3.5: *The results from repeated measure analysis (tests of within-subject and between-subjects effects) of behavioural performance between WT-P0 controls and WT-Null controls, during sessions where the ITI was altered.*

Behavioural Measures	Test of between-subject: GENOTYPE	Test of within-subject: INTERACTION
	Short ITI	
Accuracy	$F_{1, 35}$= 0.03, n.s.	$F_{2,79}$= 1.88, n.s.
Omission	$F_{1, 35}$= 0.63, n.s.	$F_{2, 82}$=1.25, n.s.
Correct RT	$F_{1, 35}$= 0.00, n.s.	$F_{2, 82}$= 0.32, n.s.
Premature nose-pokes	$F_{1, 35}$= 0.72, n.s.	$F_{2, 67}$= 0.79, n.s.
Trials	$F_{3, 35}$= 2.44, n.s.	$F_{3, 105}$= 0.92, n.s.
	Long ITI	
Accuracy	$F_{1, 35}$= 0.50, n.s.	$F_{3, 105}$= 0.70, n.s.
Omission	$F_{1, 35}$= 0.63, n.s.	$F_{2, 82}$=1.12, n.s.
Correct RT	$F_{1, 35}$= 0.03, n.s.	$F_{2, 56}$= 1.99, n.s.
Premature nose-pokes	$F_{1, 35}$=0 .60, n.s.	$F_{3, 105}$= 1.40, n.s.
Trials	$F_{1, 35}$= 0.32, n.s.	$F_{3, 89}$= 2.84, n.s.

Appendix 3.6: *The results from repeated measure analysis (tests of within-subject and between-subjects effects) of behavioural performance on the 5-CSRTT between WT-P0 controls and WT-Null controls, during sessions where the SD was altered.*

Behavioural Measures	Test of between-subject: GENOTYPE	Test of within-subject: INTERACTION
	Short SD	
Accuracy	$F_{1, 35}$= 0.17, n.s.	$F_{2, 75}$= 1.11, n.s.
Omission	$F_{1, 35}$= 1.52, n.s.	$F_{3, 105}$=2.57, n.s.
Correct RT	$F_{1, 35}$= 0.00, n.s.	$F_{2, 77}$= 2.26, n.s.
Premature nose-pokes	$F_{1, 35}$= 0.93, n.s.	$F_{3, 105}$= 0.23, n.s.
Trial	$F_{1, 35}$= 0.07, n.s.	$F_{3, 105}$= 0.24, n.s.
	Long SD	
Accuracy	$F_{1, 35}$= 0.83, n.s.	$F_{1,51}$= 0.80, n.s.
Omission	$F_{1, 35}$= 0.14, n.s.	$F_{3, 105}$= .46, n.s.
Correct RT	$F_{1, 35}$= 0.01, n.s.	$F_{3, 105}$= 0.10, n.s.
Premature nose-pokes	$F_{1, 35}$= 0.00, n.s.	$F_{3, 105}$= 0.86, n.s.

Appendix 3.6: continued (see above)		
Trial	$F_{1,35}$= 0.45, n.s.	$F_{2,71}$= 0.24, n.s.

Appendix 3.7: *The results from repeated measure analysis (tests of within-subject and between-subjects effects) of behavioural performance between WT-P0 controls and WT-Null controls, during sessions where mice gained ad libitum liquid access, 20min prior to 5-CSRTT testing.*

Behavioural Measures	Test of between-subject: GENOTYPE	Test of within-subject: INTERACTION
	***Ad libitum* condensed milk access**	
Trials	$F_{1,35}$= 0.00, n.s.	$F_{1,35}$= 0.14, n.s.
Accuracy	$F_{1,35}$= 0.15, n.s.	$F_{1,35}$= 0.43, n.s.
Omissions	$F_{1,35}$= 1.41, n.s.	$F_{1,35}$= 1.44, n.s.
Correct RT	$F_{1,35}$= 0.04, n.s.	$F_{1,35}$= 2.10, n.s.

Appendix 3.8: *The results from repeated measure analysis (tests of within-subject and between-subjects effects) of behavioural performance between WT-P0 controls and WT-Null controls, during sessions where mice were subjected to a white noise distracter stimulus (100dB) at varying intervals within the ITI.*

Behavioural Measures	Test of between-subject: GENOTYPE	Test of within-subject: INTERACTION
	White noise distracter stimulus	
Trial	$F_{1,35}$= 0.04, n.s.	$F_{3,105}$= 1.05, n.s.
Accuracy	$F_{1,35}$= 0.62, n.s.	$F_{3,105}$= 0.62, n.s.
Omissions	$F_{1,35}$= 0.01, n.s.	$F_{2,71}$= 0.62, n.s.
Correct RT	$F_{1,35}$= 0.09, n.s.	$F_{2,71}$= 0.67, n.s.

Appendix 3.9: *The results from independent T-test of behavioural measures at 1-CSRTT baseline level, between WT-P0 controls and WT-Null controls, as well as from a repeated-measures analysis of interaction between 5-CSRTT/1-CSRTT baseline sessions and genotype.*

Behavioural Measures	1-CSRTT Independent T-test	5-CSRTT/1-CSRTT Test of within-subject: INTERACTION
Trials	$t(35)$=-0.29, n.s.	$F_{1,35}$= 0.02, n.s.
Accuracy (%)	$t(35)$=-1.06, n.s.	$F_{1,35}$= 0.01, n.s.
Omissions (%)	$t(35)$=-1.02, n.s.	$F_{1,35}$= .57, n.s.
Correct RT (sec)	$t(35)$= 1.35, n.s.	$F_{1,35}$= 1.26, n.s.
Premature nose-pokes	$t(35)$=-1.43, n.s.	$F_{1,35}$= 0.18, n.s.
Magazine latency	H_1= .42, n.s.	
Eating duration	$t(35)$=-0.47, n.s.	
Nose-pokes per trial	$t(35)$=-0.98, n.s.	
Panel-pushes per trial	$t(35)$= 0.57, n.s.	

Appendix IV

Appendix 4.1: *The results of an independent T-test analysis of condensed milk reinforcer preference and total volume consumption between WT-P0 and WT-Null controls, on the final day of habituation prior to SSRTT.*

Statistical analysis	Test of between subject: GENOTYPE
Condensed milk preference	$t(28)= -0.24$, n.s.
Total volume consumption	$t(28)= -0.74$, n.s.

Appendix 6.2: *The results of an independent T-test analysis of the number of sessions required to complete each of the training phases, between WT-P0 and WT-Null controls*

Statistical analysis	Test of between subject: GENOTYPE
Single nose-poke training phase	$t(28)= 0.78$, n.s.
Go trial training phase	$t(28)= 1.30$, n.s.
Stop signal trial training phase	$t(28)= -0.90$, n.s.
SSRTT	$t(28)= 3.83$, n.s.

Appendix 4.3: *The results from repeated-measures analysis of performance of main behavioural measures at the single nose-poke phase of the SSRTT acquisition, between WT-P0 and WT-Null controls. *Significant at $p < 0.05$.*

Behavioural Measures	Repeated-measures analysis	
	Test of between-subject: GENOTYPE	Test of within-subject: INTERACTION
Trials completed %	$F_{1,28}= 1.64$, n.s.	$F_{1,28}= 0.35$, n.s.
Number of trials initiated	$F_{1,28}= 0.40$, n.s.	$F_{1,28}= 0.06$, n.s.
INIT NPs/per trial	***$F_{1,28}= 9.95$, $p= 0.005$**	***$F_{1,28}= 6.01$, $p= 0.05$**
Panel-pushes/per trial	$F_{1,28}= 2.38$, n.s.	***$F_{1,28}= 7.09$, $p= 0.05$**

Appendix 4.4: *The results from repeated-measures analysis of performance of main behavioural measures at the Go signal trial phase of the SSRTT acquisition, between WT-P0 and WT-Null controls.*

Behavioural Measures	Repeated-measures analysis	
	Test of between-subject: GENOTYPE	Test of within-subject: INTERACTION
% Go-correct	$F_{1,28}= 2.27$, n.s.	$F_{1,28}= 1.77$, n.s.
Go-correct RT	$F_{1,28}= 0.61$, n.s.	$F_{1,28}= 0.77$, n.s.
% trials completed	$F_{1,28}= 0.45$, n.s.	$F_{1,28}= 0.65$, n.s.
Number of trials init.	$F_{1,28}= 0.07$, n.s.	$F_{1,28}= 0.01$, n.s.

Appendix 4.4: continued (see above)		
INIT NPs/per trial	$F_{1,28}$= 0.18, n.s.	$F_{1,28}$= 1.41, n.s.
Go NPs/per trial	$F_{1,28}$= 0.08, n.s.	$F_{1,28}$= 0.38, n.s.
Total NPs/per trial	$F_{1,28}$= 0.01, n.s.	$F_{1,28}$= 0.99, n.s.
Panel-pushes/per trial	$F_{1,28}$= 0.00, n.s.	$F_{1,28}$= 0.12, n.s.

Appendix 4.5: *The results from repeated-measures analysis of performance of main behavioural measures at the Stop signal trial phase of the SSRTT acquisition, between WT-P0 and WT-Null controls.*

Behavioural Measures	**Repeated-measures analysis**	
	Test of between-subject: GENOTYPE	**Test of within-subject: INTERACTION**
% Stop-correct	$F_{1,28}$= 0.11, n.s.	$F_{1,28}$= 2.40 n.s.
SSRT	$F_{1,28}$= 0.07, n.s.	$F_{1,28}$= 1.26, n.s.
% Go-correct	$F_{1,28}$= 1.21, n.s.	$F_{1,28}$= 1.53, n.s.
Go-correct RT	$F_{1,28}$= 0.36, n.s.	$F_{1,28}$= 0.59, n.s.
% trials completed	$F_{1,28}$= 0.36, n.s.	$F_{1,28}$= 0.35, n.s.
Number of trials initiated	$F_{1,28}$= 1.96, n.s.	$F_{1,28}$= 0.43, n.s.
INIT NPs/per trial	$F_{1,28}$= 0.94, n.s.	$F_{1,28}$= 1.88, n.s.
Go NPs/per trial	$F_{1,28}$= 0.08, n.s.	$F_{1,28}$= 3.25, n.s.
Total NPs/per trial	$F_{1,28}$= 0.46, n.s.	$F_{1,28}$= 2.86, n.s.
Panel-pushes/per trial	$F_{1,28}$= 0.12, n.s.	$F_{1,28}$= 0.08, n.s.

Appendix 4.6: *The results of an independent T-test of the final LH values of the Go and Stop signals, between WT-P0 controls and WT-Null controls, on the final day of habituation prior to SSRTT.*

LH measure	**Test of between subject: GENOTYPE**
Go signal LH	$t(28)$=-0.11, n.s.
Stop signal LH	$t(28)$=-0.15, n.s.

Appendix 4.7: *The results from an independent T-test analysis of baseline performance of main behavioural measures on the SSRTT, between WT-P0 and WT-Null controls.*

Behavioural measures	**Test of between subject: GENOTYPE**
% Stop-correct	$t(28)$= 1.71, n.s.
SSRT	$t(28)$=-0.94, n.s.
% Go-correct	$t(28)$= 0.90, n.s.
Go-correct RT	$t(28)$=-0.36, n.s.
% trials completed	$t(28)$= -1.44, n.s.
Number of trials initiated	$t(28)$= 1.02, n.s.

Appendix 4.7: continued (see above)	
INIT NPs/per trial	$t(28)$=-0.40, n.s.
Go NPs/per trial	$t(28)$=-0.09, n.s.
Total NPs/per trial	$t(28)$=-0.25, n.s.
Panel-pushes/per trial	$t(28)$= -1.37, n.s.

Appendix 4.8: *The results of a repeated measures analysis of the magazine latency and eating duration across Go trials and Stop signal trials, at baseline, between WT-P0 controls and WT-Null controls.*

Behavioural Measures	**Repeated-measures analysis**	
	Test of between-subject: GENOTYPE	**Test of within-subject: INTERACTION**
Magazine latency	$F_{1,28}$= 0.03, n.s.	$F_{1,28}$= 1.53, n.s.
Eating duration	$F_{1,28}$= 0.16, n.s.	$F_{1,28}$= 0.54, n.s.

Appendix 4.9: *The results from repeated-measures analysis of performance of main behavioural measures across genotypes (Igf2-P0 KO, Igf2-Null lines and WT-controls) at SSRTT sessions where the SSD was varied across the Go-LH phase.*

Behavioural Measures	**One-way ANOVA: Test of between-subject effect**	
	Stop signal: 10-30% of mean Go RT	**Stop signal: 70-90% of mean Go RT**
% Stop-correct	$F_{2,51}$= 1.19, n.s.	$F_{2,51}$= 0.24, n.s.
SSRT	$F_{2,51}$= 0.83, n.s.	$F_{2,51}$=0.32, n.s.
% Go-correct	$F_{2,51}$= 0.10, n.s.	$F_{2,51}$=0 .53, n.s.
Go-correct RT	$F_{2,51}$= 0.20, n.s.	$F_{2,51}$= 0.17, n.s.
% trials completed	$F_{2,51}$= 0.77, n.s.	$F_{2,51}$= 0.22, n.s.
Number of trials initiated	$F_{2,51}$= 0.78, n.s.	$F_{2,51}$= 1.74, n.s.

Appendix 4.10: *The results from repeated-measures analysis of performance of main behavioural measures between WT-controls and WT-Null controls at SSRTT sessions where the SSD was varied across the Go LH phase.*

Behavioural Measures	**One-way ANOVA: Test of between-subject effect**		
	Stop signal: 10-30% of mean Go RT	**Stop signal: 40-60% of mean Go RT**	**Stop signal: 70-90% of mean Go RT**
% Stop-correct	$t(28)$= 1.15, n.s.	$t(28)$= 0.78, n.s.	$t(28)$= -0.32, n.s.
SSRT	$t(28)$= 0.74, n.s.	$t(28)$= 0.84, n.s.	$t(28)$= -0.36, n.s.
% Go-correct	$t(28)$=-0.56, n.s.	$t(28)$= 0.95, n.s.	$t(28)$= -0.10, n.s.
Go-correct RT	$t(28)$= 0.25, n.s.	$t(28)$= 0.37, n.s.	$t(28)$= -0.03, n.s.
% trials completed	$t(28)$= 1.85, n.s.	$t(28)$= 1.88, n.s.	$t(28)$= 1.37, n.s.
Number of trials initiated	$t(28)$=-0.66, n.s.	$t(28)$=-1.00, n.s.	$t(28)$= 0.17, n.s.

Appendix V

Appendix 5.1: *The results from a one-way ANOVA comparative analyses (of all four genotype groups) of performance between the Igf2-P0 KO mice to each of the two WT-littermate control groups, on various behavioural batteries where significant results were obtained using a collective WT-control group.*

Behavioural test:	**Behavioural parameter:**	**ANOVA significance test:**	**Post hoc comparisons between *Igf2*-P0 knockout and;**	
			WT-P0 controls:	**W-Null controls:**
Acoustic startle and prepulse inhibition	Startle response at baseline	$F_{3,80}= 7.10$, $p< 0.001$	$F_{1,56}= 12.13$, $p< 0.005$	$F_{1,30}= 18.22$, $p< 0.001$
Test of food-neophobia	Novel food-stuff preference	$F_{3,119}= 3.82$, $p< 0.05$	$F_{3,71}= 8.13$, $p< 0.05$	$F_{3,119}= 8.81$, $p< 0.05$
Elevated plus-maze	Open-arm preference	$F_{3,72}= 4.51$, $p< 0.01$	$F_{1,38}= 12.21$, $p< 0.005$	$F_{1,40}= 4.86$, n.s.
Open field	Latency of first inner-square entry	$F_{3,66}= 6.22$, $p< 0.001$	$F_{1,33}= 8.90$, $p< 0.05$	$F_{1,39}= 8.22$, $p< 0.05$
Free exploratory paradigm	Novel-compartment preference	$F_{3,65}= 3.28$, $p< 0.05$	$F_{1,39}= 4.14$, n.s.	$F_{1,35}= 7.83$, $p< 0.05$
	Latency of first distal-novel compartment entry	$F_{3,65}= 2.80$, $p< 0.05$	$F_{1,39}= 5.33$, n.s.	$F_{1,35}= 3.33$, $p< 0.05$
The 5-CSRTT	Response accuracy at baseline	$F_{3,68}= 5.67$, $p< 0.005$	$F_{1,47}= 14.67$, $p< 0.005$	$F_{1,30}= 1.77$, n.s.
	Response accuracy at Long ITI	$F_{3,68}= 5.00$, $p< 0.005$	$F_{1,47}= 6.47$, n.s.	$F_{1,30}= 8.42$, $p< 0.05$
	Response accuracy at white-noise distraction	$F_{3,68}= 2.49$, n.s.	-	-

Appendix 5.1 continued: (see above)				
The 5-CSRTT	Premature nose-pokes at baseline	$F_{3,68}$= 2.99, p< 0.05	$F_{1,47}$= 5.51, n.s.	$F_{1,30}$= 4.54, n.s.
	Premature nose-pokes at Long ITI	$F_{3,68}$= 3.92, p< 0.05	$F_{1,47}$= 7.36, p< 0.05	$F_{1,30}$= 8.93, p< 0.05

Appendix 5.2: *The results from a one-way ANOVA comparative analyses (of all four genotype groups) of performance between the Igf2-*Null *KO mice to each of the two WT-littermate control groups, on various behavioural batteries where significant results were obtained using a collective WT-control group.*

Behavioural test:	**Behavioural parameter:**	**ANOVA significance test:**	**Post hoc comparisons between *Igf2*-P0 knockout and;**	
			WT-P0 controls:	**W-Null controls:**
The 5-CSRTT	Response accuracy at baseline	$F_{3,68}$= 5.53, p< 0.005	$F_{1,36}$= 8.19, p< 0.05	$F_{1,23}$= 1.31, n.s.
	Response accuracy at Long ITI	$F_{3,68}$= 5.00, p< 0.005	$F_{1,36}$= 6.47, n.s.	$F_{1,23}$= 8.51, p< 0.05
	Omissions at Short SD	$F_{3,68}$= 2.89, p< 0.05	$F_{1,36}$= 2.96, n.s.	$F_{1,23}$= 6.88, p= 0.051