In utero adversity and later life behavioural disorders: the role of *Cdkn1c*

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Summary

Genes that are imprinted are subject to a developmentally determined epigenetic marking, which restricts expression to a single allele, dependant on the parent of origin. Selection of imprinted genes for monoallelic expression indicates their function is highly dosage sensitive. Altered dosage of imprinted genes has been linked to a number of neurological conditions, including psychosis. *Cdkn1c* is an example of an imprinted gene whose expression is sensitive to the *in utero* environment. Considerable development of the nervous system takes place *in utero* and suboptimal pregnancies have been linked to the occurrence of psychiatric and other behavioural disorders in adults. One mechanism through which the maternal environment may impact foetal development is by altering the epigenetic regulation of vulnerable genes.

A prenatal low protein or high fat diet resulted in alterations in a subset of imprinted gene in the brains of the offspring at E18.5. This was accompanied by sexually dimorphic changes in the dopaminergic system. Previously published findings reporting sensitivity of *Cdkn1c* to a prenatal low protein diet were replicated with a 1.8 fold increase in neural *Cdkn1c* expression observed. This was shown to be due to a change in the parental contribution to expression levels of this gene. Modelling the specific alteration of an increase in *Cdkn1c* genetically (*Cdkn1c*^{BACx1} line) revealed anhedonia, but with an increased motivational drive, towards a palatable solution, with corresponding changes in the reward system responsivity and chemistry in the adult brain. Additionally presence of a C*dkn1c*^{BACx1} animal in a group destabilised the social hierarchy, negatively effecting fitness of all group members.

An adverse *in utero* environment increases *Cdkn1c* levels to those reminiscent of the genetic 'loss of imprinting' model. Such alteration in expression of *Cdkn1c* has significant consequences for adult neurochemistry, reward processing and the social environment and fitness of the group. This work suggests a potentially crucial role, of at least *Cdkn1c*, and perhaps imprinted genes more generally, in mediating the negative consequences of an adverse *in utero* environment.

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Abbreviations used

5HT	Serotonin
ADHD	Attention deficit hyperactivity disorder
ASR	Acoustic startle response
BAC	Bacterial artificial chromosome
BP	Break point
BSA	Bovine serum albumin
CDKi	Cyclin dependant kinase inhibitor
CRF	Continuous reinforcement
DA	Dopamine
DMR	Differentially methylated region
Drd1	Dopamine receptor type 1
Drd2	Dopamine receptor type 2
E	Embryonic day
EPM	Elevated plus maze
ESC	Embryonic stem cell
fMRI	Functional magnetic resonance imaging
HRP	Horse radish peroxidase
i.p.	Intra-peritoneal
ICR	Imprinting control region
IUGR	Intrauterine growth restriction
LMA	Locomotor activity
lncRNA	Long noncoding RNA
LOM	Loss of methylation
LTR	Long terminal repeat
NA	Noreadrenaline
NGS	Normal goat serum
NSC	Neural stem cells
OF	Open field
PBS	Phosphate buffered saline
PGC	Primordial germ cells

PPI	Pre-pulse inhibition
PR	Progressive ratio
RT	Room temperature
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SVZ	Subventricular zone
TBS	Tris-buffered saline
VTA	Ventral tegmental area
wt	Wild type

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Chapter 1: Introduction

The current research sought to examine the effect of an altered dosage of a single imprinted gene, *Cyclin dependant kinase inhibitor 1c* (*Cdkn1c*) (also known as $p57^{Kip2}$), on brain development, neurochemistry, and adult behaviour. Additionally, the consequences of alterations to maternal diet during pregnancy on fetal brain imprinted gene expression and neural development were assessed.

1.1 Imprinted genes

Imprinted genes represent a subset of genes that, so far, are found exclusively in therian mammals and some flowering plants, which violate Mendel's second law, namely that of independent assortment. Specifically, though each gene is represented biallelically, expression of imprinted genes is monoallelic. During gametic or embryonic development one allele becomes epigenetically silenced and expression occurs predominantly from a single allele. This differs from X-chromosome inactivation in eutherian females as, rather than being (predominantly) random (Migeon et al., 1985), the identity of the silenced/expressed allele is dependent on the parent of origin of that allele.

Currently there are approximately 125 genes that are recognised as classically imprinted and show parental specific monoallelic expression (http://www.har.mrc.ac.uk/research/genomic_imprinting/). The number of loci loci displaying parent of origin effects on expression has been reported to be as high as 1300 in the mouse brain (Gregg et al., 2010). However, this figure has come under scrutiny (DeVeale et al., 2012). Regardless of the total number, what is clear is the importance of imprinted genes for embryonic development. Seminal experiments generating androgenetic, gynogenetic and parthenogenetic murine embryos revealed a non-equivalence of the parental genomes manifesting as failure of correct development of both embryonic and placental structures, and embryonic fatality (Kaufman et al., 1977; Surani and Barton, 1983; McGrath and Solter, 1984a; Surani et al., 1984; Thomson and Solter, 1988). What was particularly interesting about this series of experiments was the defined pattern of failed development for both. Embryos derived from

two copies of the maternal genome (either parthenogenetic or gynogenetic) died mid gestation (~E10) with relatively normal, if growth restricted fetal development but with poor development of extra-embryonic tissues (Kaufman et al., 1977; Surani and Barton, 1983; Surani et al., 1984, 1986; Thomson and Solter, 1988). Androgenetic embryos (derived from two copies of the paternal genome from a single donor) died at an earlier timepoint, E8.5, and were both growth restricted and developmentally delayed but with an abundance of extra-embryonic tissue (Barton et al., 1984; Surani et al., 1986; Thomson and Solter, 1988).

Further experiments were performed using embryonic stem cells (ESCs) derived from monoparental blastocysts. Detailed examination of the brains from mouse chimeras generated with parthenogenic ES cells demonstrated the necessity of representation of both parental chromosomes for neural development (Allen et al., 1995). Parthenogenetic cells were confined to specific sub-regions of the brain at embryonic and adult time-points, specifically cortical regions (including the hippocampus), superior colliuclus, red nucleus, ventral tegmental area (VTA), substantia nigra (SN), locus coeruleous, medial raphe and parabrachial nucleus with notable exclusion from the hypothalamus (Allen et al., 1995; Keverne et al., 1996). The reciprocal experiment determining the contribution of androgenic cells to the nervous system identified an almost inverse pattern, with relatively high representation of androgenetic cells in the hypothalamus and choroid plexus (Keverne et al., 1996).

Genetically more focused experiments examining uniparental disomy of specific chromosomes identified maternally inherited murine distal chromosome 7 as being crucial for development of the placental spongiotrophoblast (McLaughlin et al., 1996) and embryonic growth (Ferguson-Smith et al., 1991). Additionally, maternal or paternal disomy of proximal chromosome 11 (but not chromosome 13) of the mouse, resulted in smaller and larger offspring/placenta, respectively, compared to controls (Cattanach and Kirk, 1985; Cattanach et al., 2004). Similarly, both maternal and paternal uniparental disomy of distal

chromosome 2 resulted in perinatal lethality, with gross morphological differences from normal biparental neonates (Cattanach and Kirk, 1985).

While some imprinted genes display universally imprinted expression, some are imprinted in only a subset of tissues, while some show temporal imprinting. *Kcnq1* is imprinted in the placenta and early embryo but becomes progressively biallelically expressed (Gould and Pfeifer, 1998; Paulsen et al., 1998; Umlauf et al., 2004; Wang et al., 2011b). *Peg12* is imprinted in the embryo, adult nervous system, spleen and lung but not in the placenta (Kobayashi et al., 2002; Tran et al., 2013). *Gnas* is imprinted in adipose tissue but displays biallelic expression in the placenta and kidney (Yu et al., 1998; Okae et al., 2011). In the adult subventricular zone (SVZ), the neurogenic niche of the adult brain, *Dlk1,* imprinted in the placenta and adult brain, displays complex imprinting status. *Dlk1 is* monoallelic and biallelic in adjacent cells, the SVZ and the neural stem cells (NSCs) and niche astrocytes therein, simultaneously (Ferron et al., 2011). *Igf2* is monoallelically expressed from the paternal allele in the placenta and embryo with the exception of the choroid plexus epithelium and the leptomeninges, and the adult brain (Ohlsson et al., 1994; Pham et al., 1998). So important is correct dosage of *Igf2* that lethality of parthenogenetic embryos can, to some extent, be rescued by deletion of the *Igf2/H19* imprinting control region (ICR), restoring *Igf2* dosage (Kono et al., 2004). Possibly most interestingly of all is the case of *Grb10,* which is expressed from the maternal allele in the placenta and the majority of embryonic tissues but from the paternal allele in the nervous system (Miyoshi et al., 1998; Arnaud et al., 2003; Charalambous et al., 2003b). These parental alleles perform distinct roles in behaviour and placental function (Charalambous et al., 2010; Garfield et al., 2011; Cowley et al., 2014).

1.1.1 Evolution

The emergence of monoallelic gene expression within mammalian lineages did not occur at all imprinted loci simultaneously as indicated by differing imprinting status at the same loci between evolutionarily distant therian mammals, such as the tammer wallaby (marsupial) and mouse (eutherian) (Renfree et al., 2013). The selective pressures for monoallelic expression have

been much speculated upon (Holman and Kokko, 2013; Patten et al., 2014). One theory postulates that imprinted gene expression evolved as a method of co-adaption of interacting genes at distant loci (Wolf, 2013). Indeed, it is the case that imprinted function converges not only on the same physiological functions but also often on the same pathway or signalling cascade. For example, imprinted gene function, thus far, appears to converge on a limited number of physiological functions including fetal growth (Smith et al., 2006), metabolic function (Radford et al., 2011), placental development (Frost and Moore, 2010) and behaviour (Wilkinson et al., 2007). Even within the broad reaching topic of 'behaviour', though currently under-investigated, imprinted gene function converges on socio-sexual behaviours (Curley and Mashoodh, 2010; Úbeda and Gardner, 2010; Garfield et al., 2011; Úbeda and Gardner, 2011; McNamara and Isles, 2014), novelty reactivity (Plagge et al., 2005) and maternal care (Swaney et al., 2007; Broad et al., 2009). This convergence of imprinted gene function may arise, in some cases, from co-expression within the same cell type, as is the case for *Grb10* and *Nesp55* in the nervous system (Dent et al., in preparation). Alternatively, convergence may occur within the same signalling cascade, for example the cell cycle (Hernandez et al., 2003). G1 to S- phase transition in neural progenitors has been shown to be regulated by *Zac1* and *Cdkn1c* via *Tcf4* (Lee et al., 1995; Matsuoka et al., 1995; Schmidt-Edelkraut et al., 2013). In fact *Zac1* has been shown to directly bind the long noncoding RNA (lncRNA) *Kcnq1ot1* unmethylated promotor (Arima et al., 2005) and promote its transcription. Transcription of this lncRNA is required for monoallelic *Cdkn1c* expression (Mancini-Dinardo et al., 2006). In the opposite case, high *Igf2* expression, both *in vitro* and *in vivo*, leads to decreased *Cdkn1c* expression (Caspary et al., 1999; Grandjean et al., 2000), possibly through Igf2 binding to the tyrosine kinase receptor Igf1r and phosphorylation of Akt (Protein Kinase B). Akt has been shown to directly down regulate Cdkn1c through binding and cytoplasmic translocation (Lo, 2013; Zhao et al., 2013).

An alternative theory relates to the observation that imprinted genes tend to occur in clusters throughout the genome (Verona et al., 2003). This has led to a number of variations of the 'bystander hypothesis' of imprinted gene evolution.

The origin of this comes from the so-called 'ovarian time bomb' hypothesis put forward in the early 90's (Varmuza and Mann, 1994). This speculated that the establishment of imprinting was to prevent parthenogenesis and protect the female from hydratiform mole, or other ovarian teratoma, formation. This theory speculated that this is achieved via ovarian inactivation of genes required for trophoblast development, and that monoallelic expression spread from this initial site (Varmuza and Mann, 1994). From its inception, this theory has generated heated debate (Haig, 1994; Mann and Varmuza, 1994; Moore, 1994; Solter, 1994). Primary criticisms include that the relative rarity of malignant ovarian teratomas, especially outside of humans, was not a great enough selective pressure. Additionally the theory failed to explain the imprinting phenomenon in plants.

While the causative event for establishment of the initial imprint may be a source of contention, the 'innocent bystander' aspect to the theory is attractive and has garnered more attention that the initial theory itself. Alternative pressures for gene silencing have been since described, with the advent of whole genome sequence data. These include, as a mechanism of host defence from insertion of exogenous genetic material or retrotransposition. The idea of imprinting as a mechanism of host defence itself is an old one (Barlow, 1993). This postulates that DNA methylation arose as a mechanism to silence genomic material recognised as 'foreign' and that imprinted loci, either by similarity or proximity to this 'non-self' material also became silenced in the oocyte (Barlow, 1993). While this incarnation of the theory can now be considered incomplete, it is an exciting concept that still receives attention (Ferguson-Smith, 2011). The mammalian genome utilises DNA methylation to silence potentially harmful retroelements (Bestor, 1999, 2003; McDonald et al., 2005; Jones et al., 2010; Renfree et al., 2013). Retrotransposons are mobile genetic elements that can replicate and, though reverse transcription of their RNA, insert copies of their DNA into the nuclear genome (Havecker et al., 2004). A sub-class of these include the insertion of long terminal repeats (LTRs) which have been hypothesized as being the genome level identifier of imprinted loci as they are enriched in therian mammals compared to monotremes (Pask et al., 2009).

However, this is likely to be overly simplistic, as the increase in LTR has occurred throughout the genome, not uniquely at imprinted loci. Silencing of these elements as an initiator for the origin of imprinting remains an attractive hypothesis. Several imprinted genes have been identified as being, or speculated as being, as a result of a retrotranposition event (Pask et al., 2009; Kanber et al., 2013). A number of protein coding genes in the human 15q11-13 imprinting locus are intronless (*MAGEL2/Magel2, NDN/Ndn, Frat3/Peg12*) (Boccaccio et al., 1999; Chai et al., 2001), implying they arose from a retrotransposition event.

Microimprinted domain is a term used to describe isolated imprinted genes with few, if any, introns that are located within an intron of a biallelically expressed gene and consist of *Neuronatin* (*Nnat*), *Np1l5*, *Peg13*, *Inpp5f_v2* and *U2af1_rs1*. These are hypothesised to represent the primordial imprinted domain whereby imprinted expression may spread, or increase in complexity, from this initial site (Reik et al., 2001; Evans et al., 2005). This is concordant with the bystander hypothesis as three of these (*Inpp5f_v2*, *U2af1-rs1*, and *Nap1l5*) display characteristics of retrotransposition, lacking introns. A subset of this group (*Inpp5f_v2, Np1l5* and *Peg13)* display predominantly neural expression (Davies et al., 2004; Choi et al., 2005). If these genes do represent an early site of establishment of an imprinted locus, it implies that, at least currently, selective pressures for monoallelic expression relate to neural phenomenom and not, as has been long assumed, to placental function.

Whatever the initial event from which imprinted genes grew, parental specificity is possibly the most intriguing aspect. Were these genes simply an 'innocent bystander' to retrotransposon silencing, we would likely not observe the parental dimorphism (Haig, 2012). Why this might be the case is the source of much speculation.

1.1.1.1 The conflict theory of genomic imprinting

This theory has been given the most attention since its first description shortly after the identification of imprinted genes (Moore and Haig, 1991). Including the updated versions of the theory to account for the effects of imprinted genes on

postnatal development, a large proportion of the currently available data appears to fit this model. This centres upon the idea of a disparity, or conflict, in the interests of the maternal and paternal genomes with respect to the offspring. This theory postulates that it is in the paternal genome's best 'interest' to extract maximal resources from the mother, both *in utero* and in the pre-weaning period. This is thought to promote the likelihood of propagation of the paternal genome within a fitter offspring. This is relevant where there is a sexual dimorphism in geographical dispersal and a given female will have multiple litters, potentially with multiple fathers. Conversely, the maternal genome has equal representation in all her litters. Therefore, it is in her genome's 'interest' to limit the allocation of resources to a single litter or offspring such that more resources remain to invest in subsequent litters. As a result, there is a 'conflict' with maternally expressed/paternally silenced genes acting to limit the resources allocated to the offspring and paternally expressed/maternally silenced genes acting to maximise offspring resource extraction from the mother. The classic example of this is that of the paternally expressed, growth promoting, *Igf2* and its receptor, maternally expressed, growth restricting, *Igf2r* (Moore and Haig, 1991). Obviously these effects are subtle, as it is in a requirement for the propagation of both parental genomes' to produce a fit offspring.

This theory has been extrapolated to describe numerous aspects of offspring life, beyond the *in utero* extraction of nutrition across the placenta. Conflict theory has sought to explain an imprinted 'edge' to maternal care (Wilkins and Haig, 2003), infant sleep (Haig, 2014; Wilkins, 2014), social interactions (Haig, 2000; Úbeda and Gardner, 2010; Brandvain et al., 2011; Haig and Úbeda, 2011; Úbeda and Gardner, 2011) and mating (Wilkins and Haig, 2003). A key theme through these is the enhanced likelihood of propagation of the maternal or paternal genome through the offspring.

In utero conflict, and co-adaption, described later (Haig, 2013), are easy to conceptualise with respect to nutrient supply and demand across the placenta. However, post-natally, the predictions are less obvious. Maternal care has been

predicted to be a site at which conflict may act (Haig and Wilkins, 2000; Wilkins and Haig, 2003). Conflict may arise where there is a closer genetic relationship between offspring and the maternal line versus the paternal line, as would be the case with sex-biased (male) dispersal (Haig, 2000). A number of imprinted genes appear to fit this prediction. Females which inherit a null copy of the paternally expressed *Peg1 (Mest)* from their father display abnormal mothering behaviour (decreased latency to retrieve pups and build nests) as well as reduced placentophagia (Lefebvre et al., 1998). In addition to positively regulating suckling behaviour (Kim et al., 2013a), strikingly similar findings were observed in females with a paternally inherited null copy of *Peg3* (Li et al., 1999). This implies that *Peg1* and *Peg3* normally function to enhance maternal care. An interesting converse is the maternally expressed *Ube3a/UBE3A,* loss of which has been implicated in the aetiology of the neurodevelopmental disorder, Angelman syndrome. One of the characteristics of this syndrome is the observed positive affect that has been described to be directed primarily to the primary caregiver (Oliver et al., 2007; Mount et al., 2011). Somewhat similar observations have been made in pups with a maternally inherited loss of *Ube3a*. These pups made more vocalizations at frequencies associated with signaling to mothers to elicit care giving than wild type (wt) pups (Jiang et al., 2010). This increase was observed overall from postnatal day 8 onward and specifically when placed on the bedding carrying maternal odor cues (Jiang et al., 2010). *Ube3a* is contrasted with *Peg1* and *Peg3* as it seems to function to decrease care required by offspring, fitting with the predictions of conflict theory.

In the early pre-weaning period rodent pups maintain their body temperature through huddling and non-shivering thermogenesis (NST). Conflict theory predicts that maternally expressed genes should promote huddling and NST (Haig, 2004). Again some of the data available appears to fit this model. The *Gnas* locus contains a number of imprinted genes, at least some of which appear to influence the pre-weaning period materno-offspring interaction. *Gnasxl* has been shown to positively regulate suckling behaviour (Plagge et al., 2004). Loss of the paternally inherited copy of *Gnasxl* or *Gnas* leads to decreased brown and white adipose tissue (BAT and WAT, respectively) and results in a postnatal

growth retardation and lean bodies (Yu et al., 2000; Plagge et al., 2004). This is opposite to loss of function of the maternally expressed *Gnas,* whereby offspring have increased adiposity and become obese (Yu et al., 2000). This is an example of antagonistic effect of maternal and paternal genomes at a single genomic locus.

Other imprinted loci have been implicated in the bidirectional regulation of adipogenesis. The paternally expressed genes *Dlk1* (*Pref1*) and *Dio3* functionally converge on early life fat accumulation. Increased expression of these two genes (accompanied by deregulation of the imprinted locus) leads to failure to thrive upon switching from a high lipid (milk based) diet to the post weaning diet (Charalambous et al., 2012). These animals display a delay in accumulation of WAT and BAT and fail to survive and maintain body temperature when they transition beyond huddling (Charalambous et al., 2012). *Nnat* is a paternally expressed gene, variants in which have been linked to obesity (Vrang et al., 2010). This gene appears to negatively regulate adipogenesis as knock down promotes a BAT fate in mouse adipocytes (Gburcik et al., 2013). *Nectin* (*Ndn*) is another paternally expressed gene, knock down/out of which in mice promotes adipogenesis (Fujiwara et al., 2012). This data appears to support the conflict hypothesis, paternally expressed genes negatively regulate adipogenesis and delay offspring weaning.

Post-weaning the predictions for conflict theory have focussed on consequences for social behaviour (Isles et al., 2006; Úbeda and Gardner, 2010; Úbeda and Gardner, 2011). In an environment with a bias for male dispersal, it is predicted that maternally expressed genes should promote cooperation with a group, whereas paternally expressed genes should promote selfish, aggressive behaviours. Evidence thus far has been limited (McNamara and Isles, 2014), with the exception of the paternally expressed gene *Grb10* (Garfield et al., 2011). A paternally inherited knock out of *Grb10* increases social dominance behaviours in a tube test and barbering towards cage mates (Garfield et al., 2011). Cage mate barbering has been cited as an indicator of dominance (Long,

1972; Strozik and Festing, 1981; Haig and Úbeda, 2011), though this has come under some criticism (Curley, 2011).

However, it can not be over-looked that there are increasingly more behaviours shown to be under the influence of imprinted gene expression that have not yet been accounted for in the conflict hypothesis. The maternally expressed gene, *Nesp,* has been shown to regulate novelty reactivity (Plagge et al., 2005). Risk taking and reward seeking are two facets upon which it is conceivable to imagine a scenario involving conflict between the interests of the maternal and paternal genomes. Given the above examples and the observation that *Cdkn1c* (Joseph et al., 2003), *Igf2* (Vazin et al., 2009) and *Dlk1* (Christophersen et al., 2007; Jacobs et al., 2009; Bauer et al., 2013) have been implicated in the proliferation of dopaminergic neurons, the reward sensing neurons, it is possible that these represent sites of imprinted gene functional convergence.

1.1.1.2 Co-adaptive theory of genomic imprinting

Imprinted genes as mechanism by which a degree of adaptability can be introduced in an offspring has been speculated upon for nearly as long as imprinted genes have been known to exist (Solter, 1988; Hall, 1990; Hurst, 1997). The theory of co-adaption of mother and offspring through imprinted genes arose from two observations. First came from wt dams carrying concepti with a paternally inherited knock out of *Igf2.* These concepti, not only appear to place a decreased demand on maternal resources, they 'primed' the dam to 'invest' less in subsequent fully wt litters, as indicated by smaller litter size (Charalambous et al., 2003a). Following on from this was the finding of an additive effect on pup mortality when both mother and offspring were null for *Peg3* (Curley et al., 2004). This suggested that imprinted genes could simultaneously influence maternal care and resource demand from the fetus/pup, resulting in a maximally 'adapted' offspring (Wolf and Hager, 2006). According to this theory, maternal monoallelic expression is favoured as it enhances integration of offspring and maternal genomes and that paternally inherited genes would become silenced if maternally inherited genes were adapted to function harmoniously with the mother.

An imprinted gene that appears to fit this model is *Grb10.* A maternally inherited knock out of this gene results in large offspring (Charalambous et al., 2003b) independent of genotype of 'nurse' during the pre-weaning period (Cowley et al., 2014). Dams with a maternally inherited knock out, cross-fostered with a wt litter have smaller pups. Interestingly when *Grb10* is absent in both the mother and offspring, pups regain wt size (Cowley et al., 2014), seemingly making this gene the 'poster child' for materno-offspring co-adaption. This is complicated by the observation that the function of paternally expressed *Grb10* in the brain appears to fit with the conflict theory (Wilkins, 2014). It has been argued, and it seems likely, that these two theories are not mutually exclusive (Haig, 2013).

1.1.2 Regulation

1.1.2.1 Establishment in germ line

Germline differentially methylated regions (DMRs) that regulate monoallelic expression of an imprinted locus are termed ICRs. Regulation of monoallelic expression of clusters of imprinted genes is based upon the parental specific addition of methyl groups to cytosine residues within stretches of CpG-rich regions, known as CpG islands. These additions, during germline development, lay the foundations for subsequent imprinted gene expression. This DNA methylation (Li et al., 1993), along with repressive histone modifications and non- coding RNAs amongst others, co-operates to ensure monoallelic expression (Ferguson-Smith, 2011; Kelsey and Feil, 2013). The evolution of imprinting coincided with the appearance of novel CpG islands in the therian line (Suzuki et al., 2011a; Renfree et al., 2013), indicating correlation if not causation for imprinted gene regulation.

Genome wide demethylation occurs at approximately E11.5, as the primordial germ cells (PGCs) enter the genital ridge (Hajkova et al., 2002; Hajkova et al., 2008; Popp et al., 2010; Guibert et al., 2012; Cantone and Fisher, 2013). Differential, parental allele specific methylation at ICRs is then established during spermatogenesis or oogenesis and these germline imprint marks are protected from the genome wide demethylation that occurs post fertilisation

(Reik et al., 2001; Morgan et al., 2005), though this may not be complete (Tomizawa et al., 2011). Allele specific ICR methylation is required for correct monoallelic expression of the associated imprinted genes (Lin et al., 2003). This germ line establishment is dependent on the *de novo* methlytransferases, responsible for adding methyl groups, Dnmt3a, Dnmt3b and the co-factor Dnmt3l (Bourc'his et al., 2001; Kaneda et al., 2004; Kato et al., 2007). Dnmt3L is believed to act as a promotor of DNA methylation rather than a methyl transferase (Aapola et al., 2000; Kareta et al., 2006). Loss-of-function of Dnmt3L results in a loss of methylation at imprinted loci specifically, without an effect on global methylation levels (Bourc'his et al., 2001; Kobayashi et al., 2012). A number of factors have now been identified as being crucial for establishment of germline ICRs. The KRAB (Krüppel- associated box domain) zinc-finger protein (Zfp57) is required for establishment of methylation at the *Snprn* locus in developing oocytes (Li et al., 2008). Kdm1b (Aof1) is a histone 3-lysine demethylase that is required in oogenesis for the establishment of methylation at the *Mest (Peg1), Grb10* and *Zac1* ICRs but not the *Snrpn* ICR (Ciccone et al., 2009). *Hells* and its encoded protein Lsh, through complexes with the histone 3-lysine 9 dimethylase enzymes Glp and G9a, have been shown to be required for methylation at the *H19* ICR(Dennis et al., 2001; Fan et al., 2005).

The maintenance DNA methyltransferase enzyme Dnmt1 is required for maintenance of germline methylation marks during embryogenesis (Li et al., 1993). Additionally, it was shown that Dnmt1 acts to maintain parent of origin specific methylation in the preimplantation zygote (Hirasawa et al., 2008). *De novo* methylation during gametogenesis does not appear to be a unique feature to imprinted genes. There are known and, as yet, unknown mechanisms selectively protecting these ICRs from the post-fertilisation widespread de methylation (Kelsey and Feil, 2013). Two key molecules have been illustrated to function in this post fertilisation protective capacity. PCG7/Stella/Dppa3 has been shown to protect a subset of imprinted loci, including *Peg1*, *Peg3* and *H19*, from undergoing demethylation (Nakamura et al., 2007). This appears to be a product of its activity in preventing the conversion of methylcytosine to hydroxymethylcytosine and its the affinity for the methylated DNA associated

histone modification (Santos et al., 2005), H3K9me2 (Nakamura et al., 2012; Szabó and Pfeifer, 2012). Additionally ZFP57 has been shown to be required for maintenance of methylation at the *Dlk1-Dio3 Gtl2* ICR (Takikawa et al., 2013) as well as at *Snrpn*, *Peg1*, *Peg3*, *Peg5*/*Nnat* and *H19* DMRs (Li et al., 2008; Zuo et al., 2012). This is likely to be through complex formation with KAP1/TRIM28/TIF1 and the DNA methyltransferases (Zuo et al., 2012).

It is not clear how ICR are identified and targeted for protection from demethylation. It was hypothesized that tandem repeats in CpG islands repeats near ICR were a possible mechanism of identification of imprinted loci (Hutter et al., 2006). However, these have been shown to be both required (Yoon et al., 2001) and dispensable for monoallelic expression (Lewis et al., 2004; Mancini-Dinardo et al., 2006). Another possibility is that heritable chromatin features mark imprinted loci as it appears that histone configuration in mature sperm is non-random around retrotransposed DNA and potentially imprinted loci (Pittoggi et al., 1999; Wykes and Krawetz, 2003; van der Heijden et al., 2008).

1.1.2.2 Establishment in somatic tissue

ICRs can regulate parent of origin specific expression through at least two distinct mechanisms, CCCTC-binding factor (CTCF)-binding insulator sequences or lncRNA transcription (Fitzpatrick et al., 2002; Hikichi et al., 2003; Thakur et al., 2004; Mancini-Dinardo et al., 2006). A number of the germline maternally methylated ICRs (*Gnas*, *Kcnq1, Igf2r* and *Gpr1* loci) contain promotors for lncRNA which are active on the unmethylated paternal allele. Transcription of the lncRNA has been demonstrated to confer silencing on neighbouring paternally inherited genes for at least three loci: *Nespas*, *Kcnq1ot1* and *Air*, respectively (Fitzpatrick et al., 2002; Sleutels et al., 2002; Mancini-Dinardo et al., 2006; Williamson et al., 2006; Kobayashi et al., 2012; Kobayashi et al., 2013), and possibly also in the case of *Gpr1-Zdbf2* locus (Duffié et al., 2014). The most well studied example of imprinted gene regulation by a lncRNA is that of *Air* at the *Igf2r* locus. The ICR is methylated on the maternal allele whereas *Air* is transcribed from the paternal allele from a promoter within the ICR (Wutz et al., 1997). Genetic ablation of *Air* expression results in loss of silencing of three

maternally expressed genes, *Igf2r*, *Slc22a2* and *Slc22a3*. *Airn* overlaps with *Igfr2* (Latos et al., 2012) which suggests the possibility of transcriptional interference, but not with *Slc22a2* and *Slc22a3* suggesting a more complex mechanism (Sleutels et al., 2002). *Air* may act by recruiting repressive histone modifications to the locus (Nagano et al., 2008) as has been suggested for another regulatory lncRNA, *KCNQ1OT1* (Chiesa et al., 2012).

Four paternally methylated germline ICRs (*H19*, *Dlk1*, *Rasgrf1* and *Zdbf2* loci) have been identified all of which are intergenic and a CTCF binding/insulation mechanism has been suggested to play a role at some of these loci (Bell and Felsenfeld, 2000; de la Puente et al., 2002; Yoon et al., 2005; Woodfine et al., 2011). At the *H19* locus, the ICR contains several CTCF binding sites which, when unmethylated, bind CTCF and disrupt the interaction between downstream enhancers and the upstream *Igf2* promoter. The ICR is methylated on the paternal allele, blocking CTCF access and allowing *Igf2* transcription to occur in *cis* (Bell and Felsenfeld, 2000; Hark et al., 2000).

There are a number of additional DMRs associated with imprinted loci that are established post fertilisation during embryonic development (John and Lefebvre, 2011). These require a number of the same factors that are required for establishment of methylation at germline ICRs. These include; the DNA methyl transferase enzymes (Mohammad et al., 2010), Zfp57 (Li et al., 2008), Lsh (Fan et al., 2005) and Eed (Wang et al., 2002; Fan et al., 2005). The maternally expressed *Cdkn1c* gene is monoallelically expressed as early as E6.5 in the murine embryo (Bhogal et al., 2004) and this monoallelic expression is maintained in all tissues in which it expressed post-natally (John, personal communication). *Kcnq1*, monoallelic expression of which is controlled by the same ICR as *Cdkn1c*, transitions from mono- to biallelic expression through development, as described above. Such differences in the maintenance of monoallelic expression suggest a functional role of gene dosage into adulthood for some but not all imprinted genes.

1.1.3 Dosage

Establishing and maintaining parental specific monoallelic expression is an energetically expensive process for an organism. Additionally, this mechanism exposes the organism to an increased risk of fitness-costing recessive mutations which would, in the case of a biallelically expressed gene, normally be masked by the unmutated, dominant, allele. Therefore, it is probable that the function of imprinted genes is sensitive to correct, monoallelic, dosage and that normal development requires monoallelic expression of these genes. In line with this idea, examples of the functional consequences of improper dosage of imprinted genes can be found in human imprinting disorders. These include; transient neonatal diabetes, resulting from an over expression of paternally expressed imprinted genes from the chromosome 6q24 imprinted locus (Kant et al., 2005; Docherty et al., 2010; Laborie et al., 2010; Suzuki et al., 2010), the overgrowth syndrome BWS (Beckwith–Wiedemann syndrome), the undergrowth syndrome SRS (Silver–Russell syndrome) (Demars and Gicquel, 2012), and PWS and AS (Angelman syndrome) (Nicholls and Knepper, 2001).

In addition to human imprinting disorders, further insight into the effects of aberrant imprinted gene dosage in mammals can be gained from animal studies. Mice with a maternally inherited knock-out of the maternally expressed *Cdkn1c* gene were characterised in three studies all of which reported an absence of the predicted fetal overgrowth, based on the parental conflict hypothesis. Mice with a double dose of *Cdkn1c* were severely growth restricted from E13.5 (Yan et al., 1997; Zhang et al., 1997; Takahashi et al., 2000). When the loss-of-function model was explored in more detail, the mutant embryos were found to display fetal overgrowth at E15.5 and E18.5 in small litters but not in large litters or at term (Andrews et al., 2007; Tunster et al., 2011). The loss of growth potential in these animals may be attributed, at least in part, to significant dysfunction of the placenta (Tunster et al., 2011). Thus in this example, the dosage model was essential in identifying the growth restricting properties of *Cdkn1c*. Similarly, loss-of-function of the maternally-expressed *Phlda2* gene does not result in fetal overgrowth (Frank et al., 2002) but a model in which expression of *Phlda2* was elevated displayed late fetal growth restriction alongside significant alteration

in placental development (Tunster et al., 2010; Tunster et al., 2014). Similarly, transgenic over-expression of the paternally expressed *Dlk1* has demonstrated a dosage-related function for this gene. *Dlk1*-transgenic mice are heavier than their wild-type littermates at E16 (da Rocha et al., 2009). Postnatally, these animals fail to thrive and weigh less than wild-type animals by P14 highlighting an important role for this gene in post natal development (da Rocha et al., 2009). Deletion of the *Gtl2/Dlk1* ICR (otherwise known as the IG ICR) leading to de-repression of the paternally expressed genes at this locus (*Dlk1*, *Rtl1* and *Dio3*) and silencing of the maternally expressed genes (*Gtl2*, *Rtl1AS* and *Meg9*), results in intrauterine growth restriction (IUGR), skeletal and muscular defects and failure to thrive postnatally (Lin et al., 2007; Charalambous et al., 2012). Dosage sensitivity of imprinted genes is not restricted to mammals. In maize, over-expression of the maternally expressed gene, *Meg1*, via a non-imprinted promoter, resulted in heavier seeds with an enlarged endosperm and a bigger embryo (Costa et al., 2012).

1.1.4 Imprinted genes and brain

A number of imprinted genes are expressed monoallelically in the developing and adult brain (Isles and Wilkinson, 2000; Isles et al., 2006; Davies et al., 2007; Wilkinson et al., 2007; Keverne, 2012). The examples of *Grb10* and *Nesp* and roles in behaviour have been described above (Plagge et al., 2005; Garfield et al., 2011; Dent and Isles, 2014). This implies a possibility for a role of imprinted genes in adult behaviour, beyond what was classically predicted under the conflict hypothesis (Úbeda and Gardner, 2011). As illustrated above in the case of *Peg1/Mest* and *Peg3* imprinted genes have a role influencing mothering behaviour towards the offspring. These behavioral deficits, plus reduced milk let down, have been ascribed to the decreased number of postpartum oxytocin-positive neurons in the hypothalamus (Li et al., 1999; Champagne et al., 2009). This is attributed to the role of Peg3 in p53-mediated neonatal apoptosis, which is increased in neuroanatomical regions important for reproductive behavior, and olfactory and pheromonal processing in *Peg3*-null individuals (Broad et al., 2009). *Peg3* is also involved in regulating reproductive behaviour. Males with a loss of *Peg3* have an olfactory deficit, whereby they do not improve

reproductive performance or interest with experience (Swaney et al., 2007). This appears to be driven by deficits in the olfactory system as males carrying a null paternal allele of *Peg3* do not favour investigating sexually receptive female urine over unreceptive (Swaney et al., 2008). *Peg3*-null males do not show classic sexual experience dependent preference for estrous urine nor matched changes in immediate early gene signalling in response to these odours wild-type males do (Swaney et al., 2008).

Igf2, while not expressed at high levels in the adult brain, has been implicated in inhibitory avoidance memory retention and fear conditioning in the hippocampus and working memory by both knock down and exogenous application of this gene (Agis-Balboa et al., 2011; Chen et al., 2011; Ouchi et al., 2013). The possible mode of action of these phenotypes is through the role of *Igf2* in dendritic spine formation and maturation (Schmeisser et al., 2012). *Igf2* has also been shown to promote hippocampal neurogenesis (Agis-Balboa et al., 2011; Ouchi et al., 2013).

A paternally inherited deletion in the *Snrpn* imprinting centre results in a neural loss of expression of *Mkm3, Magel2, Ndn, Snrpn, Snod115* and *Snod116* alongside a gain in *Ube3a* expression modelling the defect reported in some Prader-Willi syndrome (PWS) patients (Chamberlain et al., 2004; Relkovic et al., 2010). Animals with this deletion have been shown to have impaired performance in a 5 choice serial reaction time task, increased startle reactivity and hyperactivity in response to palatable foodstuffs (Doe et al., 2009; Relkovic et al., 2010).

A recently emerging theme is the convergence of imprinted gene function on adult neurogenesis and NSC. *Igf2*, *Dlk1, Zac1* and *Cdkn1c* have all been shown to have a role in the neurogenic niche. *Dlk1* is expressed in monoaminergic neurons of the mid- and hindbrain, the ventral tegmental area (VTA), substantia nigra (SN), locus ceruleus (LC) and raphe nuclei of the adult rodent and human central nervous system (CNS) (Jensen et al., 2001). *Dlk1* has been shown to have a role in the regulation of the adult subventricular zone (SVZ) neurogenic niche. Dlk1 is secreted by niche astrocytes whereas the membrane bound form is

found on the surface of NSCs in the SVZ. Biparental loss-of-function of *Dlk1* reduced the number of immature NSC (Gfap, Nestin and/or Sox2 positive) and neurons in the rostral migratory stream (RMS) and olfactory bulb (OB), the fate of neurons born in the SVZ (Ferron et al., 2011). *Zac1* binds *Tcf4* regulatory units inducing *Tcf4* expression mouse embryonic stem cells (ESCs) undergoing neuronal differentiation (Schmidt-Edelkraut et al., 2014). *Tcf4* then, via *Cdkn1c*, promotes cell cycle exit and neural differentiation (Schmidt-Edelkraut et al., 2014). Additionally, *Zac1,* in mouse ESCs directed to astroglial fate, blocks inappropriate astroglial development via direct DNA binding to the regulatory domain and activation of *Socs3* (Schmidt-Edelkraut et al., 2013). Zac1 and Socs3 additionally co-localise in the developing neocortex *in vivo* (Schmidt-Edelkraut et al., 2013), implying this *in vitro* role may be functionally relevant. Finally, in the adult, similar to *Dlk1*, *Cdkn1c* is expressed in the neurogenic niche of the SVZ and the hippocampal subgranular layer (SGL) in the adult rat and mouse brain (Jadasz et al., 2012; Furutachi et al., 2013). Its function here appears to be maintaining quiescence of adult NSCs (Furutachi et al., 2013). Conditional deletion of *Cdkn1c* in immature (Nestin positive) neurons in adulthood results in an initial increase of NSC proliferation with a later failure to induce new neurons after induction (Furutachi et al., 2013). *Cdkn1c* has been linked to the positive effect on neurogenesis of antidepressants (Anacker et al., 2011), highlighting the importance and multi-functionality of this gene, discussed in further detail below.

1.1.5 Cdkn1c

Cdkn1c is a maternally expressed imprinted gene located in the *Kcnq1* imprinting locus on mouse distal chromosome 7/human chromosome 11p15. It is monoallelically expressed as early as E6.5 in the murine embryo and its promoter acquires differential DNA methylation subsequently, at around E9.5 (Bhogal et al., 2004). *De novo* DNA methylation appears to be required for *Cdkn1c* to be expressed from the maternal allele as loss of function of the Dnmt3a results in biallelic silencing (Kaneda et al., 2004). Conversely, maintenance of DNA methylation appears to be required for paternal silencing

as loss of function of Dmnt1 results biallelic expression of *Cdkn1c* in the E9.5 embryo and ectoplacental cone (Caspary et al., 1998). Mouse chromosome 7 possesses a number of distinct imprinted domains (Wood and Oakey, 2006) and imprinting of these did not occur concurrently (Ager et al., 2008). Phylogenetic analysis showed that though the function of both *Cdkn1c* and *Igf2* are conserved between marsupials and eutherian mammals but in the marsupial lineage (tammar wallaby) only *Igf2* is imprinted (Ager et al., 2008) whereas *Cdkn1c* and the nearby *Phlda2* gene are both biallelically expressed in marsupials (Suzuki et al., 2005; Suzuki et al., 2011b). The CpG island which constitutes the ICR of the *Kcnq1* locus is also absent in the marsupial genome (Ager et al., 2008).

The ICR locus associated with *Cdkn1c*, *KvDMR* aka *IC2*, is a CpG island located in intron 10 of the *Kcnq1* gene, and contains the promoter for the lncRNA *Kcnq1ot1* (Mancini-DiNardo, 2003). This site is a germline DMR and is methylated specifically in oocytes, not sperm (Engemann et al., 2000). Transcription from the paternal allele of this lncRNA is required for silencing in *cis* and maternal monoallelic expression of the surrounding genes (Fitzpatrick et al., 2002). Paternal inheritance of a deletion of the KvDMR, including the promoter of *Kcnq1ot1,* leads to derepression and biallelic expression of *Cdkn1c* in the fetal liver, as well as other maternally expressed genes at the locus (*Tssc3/Phlda2*, *Slc22a1l, Kcnq1*, *Tssc4* and *Ascl2)* and this results in fetal growth restriction (Fitzpatrick 2002). Paternal inheritance of a deletion of the KvDMR also results in loss of *Kcnq1ot1* and further work established that the lncRNA itself is required to establish silencing of the Kcnq1 domain (Mancini-Dinardo et al., 2006). One study suggested that the lncRNA acts to bring the *Kcnq1* promotor into proximity with the KvDMR in *cis*, alongside the recruitment of polycomb repressive complex 2 resulting in the silencing of *Kcnq1* (Zhang et al., 2014). Though the full length *Kcnq1ot1* was required for *Cdkn1c* silencing in the placenta and fetal heart, brain and gut, only partial loss of silencing occurred in the liver, kidney and lung (Shin et al., 2007). This suggests that either the truncated *Kcnq1ot1* transcript possesses some function or that a more complex mechanism regulates *Cdkn1c* imprinting. The insulator CTCF was found to bind upstream of the paternally inherited unmethylated KvDMR (Fitzpatrick et al.,

2007) which may suggest silencing of *Cdkn1c* in a manner similar to the *H19* locus. It is known that there are tissue specific enhancers/silencers that lie more than 315 kb from the gene (John et al., 1999; John et al., 2001) but within 880 kb (Cerrato et al., 2005). Further work is required to determine the detailed mechanism.

1.1.5.1 Function

Cdkn1c (aka $p57^{Kip2}$) was originally identified based on similarity with p21 and p27 and these three genes now form the Cip/Kip family of cyclin dependant kinase inhibitor (CDKi) (Lee et al., 1995; Matsuoka et al., 1995). *Cdkn1c* acts to block G1 to S-phase transition (Matsuoka et al., 1995) with inhibitor activity on cyclin E/Cdk2, cyclin A/Cdk2, cyclin E/Cdk3, and cyclin D2/Cdk4 kinase complexes *in vitro* and direct binding of Cdk2 *in vivo* (Matsuoka et al., 1995; Joaquin et al., 2012; Cerqueira et al., 2014; Ishino et al., 2014). Shortly thereafter, *Cdkn1c* was mapped to the mouse distal 7/human 11p15 domains and reported to be imprinted and maternally expressed in both human and mice (Hatada and Mukai, 1995; Hatada et al., 1996b; Matsuoka et al., 1996). Critically, *CDKN1C* was found to be mutated in some BWS patients (Hatada et al., 1996a) leading to a considerable body of research to determine the function of this gene.

1.1.5.2 *Cdkn1c* in BWS and SRS

BWS and SRS are over/under-growth syndromes, respectively, which are associated with dysregulation of the human 11p15 imprinted locus. This is a complex locus containing two distinct imprinted domains one spanning the paternally expressed growth factor *Igf2* and one spanning *Cdkn1c*. The ICR1/H19 DMR controls the transcription of the paternally expressed growth factor, *Igf2* (Thorvaldsen et al., 1998). As described earlier, the ICR2/KvDMR and the paternally expressed lncRNA, *KCNQ1OT1*, regulate parent of origin specific expression of a set of maternally expressed of genes, including *Cdkn1c*. Loss of methylation at the KvDMR and biallelic expression of *KCNQ1OT1* is the most alteration observed in BWS (~50% of cases) (Lee et al., 1999; Chiesa et al., 2012).

Recent studies have also identified, a BWS patient with a microdeletion containing the KvDMR (Algar et al., 2011). Other alterations associated with BWS include paternal uniparental disomy (Weksberg et al., 2001), deletion of the regulatory *KCNQ1OT1* transcript (Niemitz et al., 2004), hypermethylation of the H19 DMR with biallelic expression, of *Igf2* (Weksberg 2001 HMG) and microdeletion of the H19 CTCF-binding sites (Sparago et al., 2004; Demars et al., 2014) (reviewed in (Weksberg et al., 2005; Enklaar et al., 2006)). Although expression levels of imprinted genes in the region have rarely been examined, studies from the mouse predict that these alterations would result in either a net increase in the growth promoting *Igf2* transcript or a decrease *Cdkn1c* or a combination of these defects.

The (epi)genetic nature of SRS are less well defined compared to BWS. Like BWS, SRS is both phenotypically and genetically heterogeneous. Maternal uniparental disomy of 11p15 has been reported in some patients (Bullman et al., 2008) (Kotzot et al., 1995) (reviewed in (Eggermann et al., 2010)). Approximately 40% of SRS cases display hypomethylation at H19-DMR (Gicquel et al., 2005; Peñaherrera et al., 2010; Turner et al., 2010; Begemann et al., 2011) occasionally associated with biallelic H19 and reduction in *Igf2* (Gicquel et al., 2005) while changes in methylation at KvDMR are rarely observed (Turner et al., 2010; Begemann et al., 2011). Recent studies have reported maternally inherited duplication of the region containing the KvDMR in patients with SRS (Schönherr et al., 2007; Bonaldi et al., 2011; Chiesa et al., 2012). Conflicting evidence has been reported on the effect of paternally inherited deletion of the centromeric 11p15 imprinted locus, with both severe growth restriction (De Crescenzo et al., 2013) and no gross phenotype (Algar et al., 2011) being reported. It seems counterintuitive that LOM at ICR2 can result in two syndromes with such different clinical presentations, however it has been suggested (Azzi et al., 2009; Begemann et al., 2011) that this may be as a result of epigenetic tissue mosaicism, seen in the differential methylation status between buccal, blood and fibroblasts in patients with SRS (Begemann et al., 2011). Mutations in *Cdkn1c* have recently also been observed in patients with IMAGe syndrome (Arboleda et al., 2012), a complex disorder whose early

clinical features, including IUGR, overlap with that of SRS. A number of patients with BWS or SRS present with loss of imprinting at multiple loci (Azzi et al., 2009; Turner et al., 2010; Kannenberg et al., 2012) which implicates a potential imprinting deficient in these syndromes with a particular sensitivity at the 11p15 locus.

1.1.5.3 Loss of function

Studies examining a maternally inherited loss of *Cdkn1c* reported increased cell proliferation alongside a number of skeletal and muscular defects with some similarities with BWS (Yan et al., 1997; Zhang et al., 1997; Takahashi et al., 2000; Tateishi et al., 2012) . No fetal overgrowth was reported in these studies and the transgenic pups die shortly after birth attributed to difficulty sucking due to presence of a cleft palate (Yan et al., 1997; Zhang et al., 1997). While these early studies failed to identify a fetal overgrowth phenotype, a more recent study examining fetal weights from E13.5 onwards reported early overgrowth that was not maintained until term (Andrews et al., 2007; Tunster et al., 2011). Importantly, in this study fetal overgrowth was only apparent later in gestation in litters with few foetuses present. Loss of function of *Cdkn1c* also resulted in placentomegaly but with very severe malformation particularly of the labyrinth zone (Andrews et al., 2007; Tunster et al., 2011). Human pregnancies differ from murine in average number of offspring, humans on the whole having singleton births, and mice having multiple births. This suggested that, when intrauterine competition is low (i.e. when litter size is small) embryos lacking maternally inherited *Cdkn1c* display an overgrowth phenotype similar to that observed in BWS patients (Andrews et al., 2007; Tunster et al., 2011) but this growth advantage is not maintained when competition is high, possibly due to the placental defects (Tunster et al., 2011).

1.1.5.4 Gain in expression/BAC Transgenic models

In order to explore to consequences of increased *Cdkn1c* dosage, a novel model was developed which carried additional copies of the *Cdkn1c* gene on a bacterial artificial chromosome (BAC). Mice were generated carrying an 85 kb BAC transgene spanning *Cdkn1c, Phlda2* and *Slc22a18* (John et al., 2001). The BAC

did not recapitulate the full expression profile of *Cdkn1c*. Transgene expression was restricted primarily to neural tissues, lung endothelium and the developing kidney tubules, where expression overlapped with endogenous *Cdkn1c*. In contrast, the BAC derived *Cdkn1c* was not expressed in the skeletal muscle, cartilage or placenta, normal sites of *Cdkn1c* expression (John et al., 2001; Andrews et al., 2007). Despite this incomplete expression profile, several BAC lines carrying increasing copy numbers of the BAC displayed marked embryonic growth restriction on a mixed 129/MF1 background and, to a lesser extent, in a mixed 129/C57BL/6 background (Andrews et al., 2007; Tunster et al., 2010). Line 5D3 (single additional copy of the BAC hereafter referred to as *Cdkn1c*^{BACx1}) and line 5A4 (two additional copies hereafter referred to as *Cdkn1c*^{BACx2}) both displayed fetal growth restriction without postnatal catch-up (Andrews et al., 2007). Growth restriction was symmetrical with no significant difference in organ weight between those overexpressing *Cdkn1c* (brain and kidney) and those where endogenous *Cdkn1c* alone was expressed (Andrews et al., 2007). This may be due to decreased *Igf1,* which acts globally to regulate fetal growth. Importantly these effects were not seen in a control BAC line carrying three copies of the same BAC but modified such that *Cdkn1c* was not expressed from the BAC (Andrews et al., 2007).

Taken together, these mouse models demonstrated a potent role for *Cdkn1c* as a negative regulator of fetal growth potentially explaining the phenotype observed in BWS and SRS patients with loss-of and gain-in-expression of *CDKN1C*, respectively.

1.1.5.5 Neural Function

In addition to a more global role in regulating fetal growth and placental development, numerous studies have identified more specific functions for *Cdkn1c* in the nervous system. *Cdkn1c* is expressed in the developing nervous system with abundant expression at E11.5 and E13.5 particularly in the developing neural fold (Westbury et al., 2001). More detailed neural analysis showed *Cdkn1c* expression to be located to zones of neurogenesis, specifically the ventricular, mantle and SVZs, from which cortical neurons are born and

migrate dorsally to the their final neocortical location (Ye et al., 2009). *Cdkn1c* was present in approximately 52% of hypothalamic neurons during proliferation (E14.5) but not BrdU positive neurons (Ye et al., 2009). This implied a role for *Cdkn1c* in neurons as they exit the cell cycle and begin to differentiate (Gui et al., 2007). Down regulation of *Cdkn1c* after knock out of *nucleosome assembly protein 1-like 2* (*Nap1l2)* in mice, likely as a result of increased histone 3-lysine 9 and 14 acetylation, is hypothesized to be the cause of the observed overproduction of neural progenitors and failure of neural tube closure in these mice (Rogner et al., 2000; Attia et al., 2007). Interestingly, a secondary role for *Cdkn1c* in cortical migration has been described that is independent of effects of differentiation. RNAi mediated knock down of *Cdkn1c* at E14 impaired neocortical neuronal migration without an effect on differentiation or on radial glia (Itoh et al., 2007; Tury et al., 2011), though this may be as a result of effects on the related *Cdkn1b* (*$p27^{kip1}$*) (Nguyen et al., 2006). Cortical neuron progenitors cultured from a *Cdkn1c* knock out (KO) embryo more frequently had increased process length (Tury et al., 2011) implying a role for *Cdkn1c* in neuronal morphology. Given that neurons over produce processes that are selectively pruned in an activity dependant manner (Zhang and Poo, 2001), it is possible this observation is as a consequence of aberrant pruning rather than a growth promotional function.

A number of the effects of *Cdkn1c* on neural development are independent of its N-terminal cell cycle domain (Tury et al., 2011). The direct transcriptional repressive activity of Cdkn1c on Mash1 remained intact after mutation of the N-terminal cyclin dependent kinase (CDK) binding domain (Joseph et al., 2009). This interaction, observed at E12.5 in the developing mouse brain, inhibits neural differentiation (Joseph et al., 2009), possibly to prevent precocious neurogenesis (Yan et al., 1997; Dyer and Cepko, 2000). Additionally, Cdkn1c cooperates with Nurr1, by direct interaction with Nurr1 N-terminal, to promote the proliferation of midbrain dopaminergic neurons (Joseph et al., 2003). At E13.5 *Cdkn1c,* unlike *Cdkn1a* or *Cdkn1b,* expression in the developing midbrain overlaps with *Nurr1* and *Th.* Embryos null for maternally inherited *Cdkn1c* display reduced numbers of Nurr1 positive and Th positive (a marker for

dopaminergic neurons) cells specifically in the ventral midbrain at E18.5 , but not E13.5 (Joseph et al., 2003). This link between *Cdkn1c* and dopaminergic cell fate has been replicated *in vitro* in human ESCs (Freed et al., 2008).

1.2 Dopaminergic system

There are four main dopaminergic tracts in the mammalian brain; the nigrostriatal pathway (movement), the mesocortical and mesolimbic tracts (emotion and motivation) and the tuberoinfundibular tract (hormone secretion) (Schwartz, 2000). *Cdkn1c* from the BAC transgene in *Cdkn1c*$^{\mathrm{BACx1}}$ animals is expressed in the developing midbrain from which the SN pars compacta (SNc) (A9) and VTA (A10) arise (Dahlstroem and Fuxe, 1964; Smidt and Burbach, 2007). These two nuclei project to the striatum and forebrain and have well characterised effects of motor circuitry and motivation, respectively, though some functional overlap does occur (Wise, 2009). All catecholamine transmitters (dopamine, noradrenaline and adrenaline) are synthesized from a common precursor, tyrosine, by the rate limiting enzyme tyrosine hydroxylase. This produces L-DOPA which is then decarboxylated to form dopamine (Schwartz, 2000). Noradrenergic neurons are characterised by the presence of dopamine β-hydroxylase on the membrane of the presynaptic vesicles where dopamine is converted to noradrenaline (Schwartz, 2000). Dopamine is loaded into presynaptic vesicles in preparation for release into the synaptic cleft by activity of the vesicular monoamine transporter 2 (Erickson et al., 1992). After neurotransmitter release dopamine is removed from the synaptic cleft by the activity of the pre-synaptic membrane bound dopamine transporter (Dat) (Schwartz, 2000). The stimulant amphetamine acts on Dat as a competitive inhibitor of dopamine reuptake, prolonging dopamine activity on its receptors (Jones et al., 1998). There are two main families of dopaminergic receptors, D1 and D2, based on their structural similarities and intracellular messengers. DRD1 and DRD5 make up the D1-like group and couple to Gαs intra-cellularly and increase cAMP levels (Dearry et al., 1990; Zhou et al., 1990; Grandy et al., 1991; Sunahara et al., 1991). DRD2, DRD3 and DRD4 make up the D2-like group and couple to Gαi intra-cellularly and decrease cAMP levels (Bunzow et al., 1988; Dal Toso et al., 1989; Sokoloff et al., 1990; Van Tol et al., 1991). DRD1 and

DRD2 are the most abundantly expressed dopaminergic receptors in the human brain (Hurley and Jenner, 2006). DRD2 has two splice variants, a long and short form with the short form predominantly acting as an autoreceptor located on the presynaptic terminal (Sesack et al., 1994; Haber et al., 1995) and the long form acting as a postsynaptic receptor (Usiello et al., 2000). DRD1 and DRD2 receptors also differ with respect to their binding capacity for dopamine, with DRD2 having a greater affinity for dopamine than DRD1 (Baik, 2013). This means that different receptors can be activated differentially depending on whether dopaminergic cell firing is phasic or tonic, introducing flexibility within the system.

1.2.1 Motor circuitry

Dopamine released from the SNc activates two receptor subtypes on the postsynaptic neuron in the striatum, specifically in the putamen. These postsynaptic medium spiny neurons (MSNs) are GABAergic and are activated or inhibited depending on whether dopamine receptor type 1 (Drd1) or dopamine receptor type 2 (Drd2), respectively, is stimulated (Gerfen et al., 1990). From this the direct (Drd1) or indirect (Drd2) pathways provide positive or negative feedback to the thalamus, respectively, and ultimately the motor cortices (DeLong, 2000). Activity of the striatum has the net effect of reducing thalamo-cortical inhibition and, therefore, facilitating movement initiation (DeLong, 2000). Activity in this circuitry that is reduced in Parkinson's disease, as a result of dopaminergic cell death in the SNc, resulting in the characteristic akinesia (Redgrave et al., 2010). This classical view of basal ganglia control is likely to be overly simplistic. It is now understood that there is a much greater degree of interconnectivity between nuclei (Redgrave et al., 2010). Additionally, cortical projections from the basal ganglia (consisting of the striatum, globus pallidus, substantia nigra and the subthalamic nucleus) are not only involved in movement initiation. Bilateral basal ganglia activity has been observed, using functional magnetic resonance imaging (fMRI), in human subjects performing tasks involving emotion, reward, working memory and executive function (Arsalidou et al., 2013). This highlights the functional heterogenetity of the basal ganglia output.

1.2.2 Reward circuitry

A10 (VTA) dopaminergic neurons project predominantly to the ventral striatum, cortical and limbic areas. They are involved in the regulation of emotion and natural reward. With respect to reward "wanting" the mesolimbic pathway consisting of VTA projections to the nucleus accumbens has been the best characterised (Berridge, 1996; Baik, 2013). The VTA receives a number of modulatory inputs including projections from the cortex, core and shell of the nucleus accumbens, dorsal striatum, pallidum central nucleus of the amygdala, lateral hypothalamic area, zona incerta, parasubthalamic nucleus, periaqueductal grey, dorsal raphe nucleus and the parabrachial nucleus of the hindbrain (Watabe-Uchida et al., 2012). This suggests a role for a range of neurotransmitters, neuropeptide and other signalling molecules in regulating reward processing.

The mesolimbic and mesocortical dopaminergic tracts are also the site of action of a number of drugs of abuse (Wise, 1998). LTD at glutamatergic synapses in the VTA, described above, is impaired in the presence of amphetamine (Jones et al., 2000). VTA projections to the nucleus accumbens in the ventral striatum undergo changes in connectivity in response to prolonged exposure to drugs of abuse, resulting in dependence and addiction (Nestler, 2001). Dopamine released onto neurons in the nucleus accumbens stimulates an increase in cAMP, CREB phosphorlyation and CREB binding protein (CBP) mediated transcription induction (Guitart et al., 1992; Nestler, 2001). Overexpression of CREB in the nucleus accumbens decreases the rewarding properties of cocaine as indexed by a conditioned place preference (CPP) task (Carlezon et al., 1998). Regulation of the Fos family of transcription factors is thought to underlie the transition to addiction (Nestler et al., 2001). A number of these genes (*c-Fos, FosB, Fra-1, Fra-2*) are rapidly, but transiently, induced in the nucleus accumbens after exposure to an addictive substance (Hope et al., 1994). *c-Fos* induction reduces in intensity after repeated administrations of cocaine (Moratalla et al., 1996). ΔFosB, though only weakly induced by cocaine, is particularly stable (Chen et al., 1997) and accumulates upon repeated administration (Moratalla et al., 1996). Induced expression of ΔFosB induced

expression in the nucleus accubens increases sensitivity to low doses of cocaine in a CPP and a locomotor activity task in mice (Kelz et al., 1999). The plasticity of the mesolimbic and mesocortical systems may underlie the variability in vulnerability to developing drug addiction and other neuropsychiatric disorders associated with this circuit, including schizophrenia (Keshavan et al., 2008) and depression (Nestler and Carlezon Jr, 2006).

1.2.3 Hormone secretion

Dopaminergic neurons in the dorsomedial arcuate nucleus in the mediobasal hypothalamus inhibit prolactin secretion from the anterior pituitary (MacLeod and Lehmeyer, 1974) by binding to Drd2 receptors on the prolactin producing lactotrophs (Lyons and Broberger, 2014). Prolactin has numerous functions in relation to lactation stimulation and reproduction (Lyons and Broberger, 2014). These include promotion of maternal behaviour (Bridges et al., 1985), stimulation of increased food intake (Sauve and Woodside, 1996), increased fat storage through leptin resistance (Augustine and Grattan, 2008; Trujillo et al., 2011), neurogenesis in the SVZ (Shingo et al., 2003) and fertility (Devi and Halperin, 2014). Many typical antipsychotics, including hapoperidol, induce hyperprolactinaemia (Esel et al., 2001), generating a slew of unwanted side effects. This is as a result of antagonism of the Drd2 receptor (Stone et al., 2009).

1.2.4 Role of imprinted genes

In addition to *Cdkn1c, Igf2* and *Dlk1* described above, other imprinted genes have been implicated in the development of the dopaminergic system. *Grb10* has been shown to be expressed in the developing ventral midbrain at E14.5, in Dat positive neurons in the adult SNc and in the VTA (Garfield et al., 2011). More generally, as mentioned above, in chimeric embryos parthenogenetic ESCs contribute to a subset of neural regions, including the VTA and SNc (Allen et al., 1995; Keverne et al., 1996). More recently, the midbrain, including the VTA and SNc was identified as an imprinting 'hotspot' in the mouse brain (Gregg et al., 2010). It is plausible, therefore, that imprinted gene functionally converge on reward related behaviours.

1.4 In utero environment

1.4.1 Effects on postnatal behaviour

Numerous studies, in both rodent and human, have linked suboptimal *in utero* environments to adverse outcomes in the offspring. A host of bodily systems have been found to be compromised, from cardiovascular (Barker, 1995) to metabolic function (Entringer, 2013), but of particular interest here are the effects on offspring behaviour (Bale et al., 2010). In humans, maternal stress (Meijer, 1985; Wadhwa et al., 1993; Weinstock, 2008), poor diet (Brown et al., 2000), depression (Rice et al., 2007), drug use (Roussotte et al., 2011; Buckingham-Howes et al., 2013) and/or alcohol use (Jacobson and Jacobson, 2002; Mattson et al., 2006) during pregnancy have been linked to a host of abnormalities. These include, but not limited to, delayed fetal growth, neural structural alterations, impaired cognitive performance, social abnormalities and an increased risk of psychiatric illnesses later in life (Huttunen and Niskanen, 1978; van Os and Selten, 1998; Brown et al., 2000; Richards et al., 2001; Laplante et al., 2004; Malaspina et al., 2008; Sandman et al., 2011). This is paralleled by work in rodents whereby prenatal stressors, mimicking human adverse *in utero* environments, cause a number of behavioural (Abel, 1982; Fride et al., 1986; Ward and Stehm, 1991; Keshet and Weinstock, 1995; Jones et al., 2010; Cullen et al., 2013) (Zagron and Weinstock, 2006; Vucetic et al., 2010a; Vucetic et al., 2010b; van den Hove et al., 2011; Vucetic et al., 2011; Vucetic et al., 2012; Carlin et al., 2013; Hausknecht et al., 2013; Sanchez Vega et al., 2013) Poon 2013, (Grissom et al., 2013; Poon et al., 2013) and neural abnormalities (Berger et al., 2002; Van den Hove et al., 2006; Hausknecht et al., 2013). The wide variety in outcomes may be a reflection of the fetal age at of exposure as well as the nature of the stresser.

Additionally important to consider are the trans-generational effects of an adverse *in utero* environment. The primordial germ cells are formed *in utero* and consequences resulting from manipulations to the *in utero* environment have been observed in the F2 generation (Radford et al., 2012; Radford et al., 2014). In fact, it is not singly the maternal environment during gestation that

can affect offspring behaviour. Spermatogenesis continues in the adult male, therefore the male environment has the potential to impact sperm development in the adult. It has been shown, in rodents, that paternal alcohol ingestion, both acute and sustained, in the absence of any maternal environmental abnormalities, results in offspring hyperactivity (Abel, 1993), deficit in passive avoidance training (Abel, 1994) and spacial learning (Wozniak et al., 1991).

The consequences on offspring behaviour of a suboptimal *in utero* environment are varied. Rodents exposed to stress prenatally display a heightened sensitivity to hepatic beta-adrenergic receptor stimulation by isoprenaline later in gestation (Slotkin et al., 1994) and additionally have been shown to have 2.5 fold increase in CRF binding sites in the amygdala compared to control animals (Ward et al., 2000). In rodents, though prenatal stress is associated with elevated corticosterone release on postnatal day 1 (P1), this has been shown to be reversed by P8, implying an enhanced ability to cope with stress exposure (Post, 1992). A nutrient restricted environment *in utero* causes early pubertal onset in female rats, irrespective of postnatal diet (Sloboda et al., 2009). This could allow greater reproductive success compared to a normal age pubertal onset in a challenging environment which would be advantageous. Interestingly, early pubertal development, in one study, has recently been linked to polymorphisms in imprinted domains (Perry, 2014). Additionally, though significantly smaller than those exposed to a standard diet *in utero*, offspring exposed to gestational malnutrition when fed *ad libitum* are heavier, with increased food intake, than control animals from P30 onwards (Manuel-Apolinar et al., 2010). This is consistent with fetal programming of these animals to store energy reserves when available, potentially anticipating a future nutrient poor environment. This system could programme the offspring for the postnatal environment, such that their development is as efficient as possible. These studies are consistent with the hypothesis that changes seen in animals in response to an environmental challenge appear to be adaptive in nature (Bateson et al., 2004).

This priming effect assumes all gestational environmental conditions encountered are natural ones. This flexibility in developmental trajectories can be hijacked by non-natural stimuli such as 'junk-food' or drugs of abuse. For example, offspring of rodent dams fed palatable foods during pregnancy and lactation and maintained on this diet into adulthood displayed an enhanced preference for food rich in fat, sugars and salt, compared to those exposed to a 'junk food' diet during lactation and/or gestation alone (Bayol et al., 2007). Additionally, human children exposed to methamphetamine *in utero* at two years had an elevated cortisol response after separation from their caregiver, especially if the caregiver reported a high potential for physical abuse (Kirlic et al., 2013). This could be a consequence maladaptive programming of the fetus for the post natal environment based on prenatal insults.

1.4.2 Imprinted gene sensitivity

The *in utero* environment is critical for the development of the nervous system. The expansion in complexity requires precise gene expression, for often restricted time windows and in discrete locations, and perturbations in this can have severe consequences for the foetus. Imprinted genes, in both human and rodent studies, have been shown to be sensitive in a number of tissues, *in utero* to various conditions including maternal dietary fat (Gallou-Kabani et al., 2010; Lin et al., 2012; King et al., 2013), fibre (Lin et al., 2012), protein (Gong et al., 2010; Vucetic et al., 2010b; Ivanova et al., 2012; Claycombe et al., 2013), nutrient restriction (Broad and Keverne, 2011; Radford et al., 2012; Radford et al., 2014), IUGR (McMinn et al., 2006; Guo et al., 2008; Cordeiro et al., 2014), bisphenol A exposure (Susiarjo et al., 2013) and maternal glucocorticoid treatment (Drake et al., 2011) (see table 1.1). Some of these effects can be detected in the F2 generation (Drake et al., 2011; Radford et al., 2012; Radford et al., 2014). The function of the plasticity of these gene in response to the *in utero* environment has been speculated on previously with respect to control of energy balance (Charalambous et al., 2007; Radford et al., 2011), but given the role of imprinted genes in the brain, as discussed above, this plasticity may affect adult behaviour of the offspring.

However, plasticity of imprinted gene expression may not be a universal phenomenon. Radford and colleagues (Radford et al., 2012) showed in an unbiased general screen that placental and hepatic expression of imprinted genes was neither more nor less susceptible to an environmental insult, relative to the transcriptome as a whole. Instead, a subset of imprinted genes appeared to moderate offspring response to this particular insult, namely maternal under-nutrition. However, like all experiments of this nature, this study has limitations, for instance only one environmental manipulation was examined and exposure was within a limited time-window (embryo day (E) 12.5-18.5). Moreover, there was no assessment of neural transcriptome in this manner; therefore it is possible that a key subset of imprinted genes in the brain may also programme the offspring's adult behaviour as consequence of this environmental challenge, tweaking to suit the environment. Nonetheless, what the Radford *et al.* (Radford et al., 2012) study highlights is that there is not a "one size fits all" profile of imprinted genes affected by gestational and/or early life adversity. Genes that are altered differ by tissue and nature of the insult, possibly reflecting differential sensitivity of the upstream regulators of individual genes. Expression of these genes may either be sensitive to, insensitive to or protected against changes in maternal diet during pregnancy. These changes, if any, may or not cause alterations in the neural development and could explain some of the behavioural alteration observed in offspring exposed to gestational adversity.

Table 1.1: Prenatal and early life conditions shown to alter imprinted gene expression in the offspring (FO: females only, MO: males only)

Maternal condition	Tissue	Gene	Time point	Direction of effect	Species	Reference
Maternal high fat	Placenta	*Slc22a2*	E15.5	↑	Mouse	(Gallou-Kabani et al., 2010)
		Slc22a3	E15.5	↓		
		Rtl1	E15.5	↓		

		Dio3 *Dlk1*	E15.5 E15.5	↓(FO) ↓(FO)		
	Placenta	*Igf2* *Igf2r* *Slc38a4*	E14.5 E14.5 E14.5	↑(MO) ↑(MO) ↑(FO)	Mouse	(King et al., 2013)
Post-weaning high fat	Sub-cutaneous adipose tissue	*Igf2*	P84	↑(MO)	Mouse	(Claycombe et al., 2013)
Maternal high sugar- high fat	Placenta	*Igf2* *Dlk1* *Snrpn* *Grb10* *H19*	E16 E16 E16 E16 E16	↑ ↑ ↑ ↑ ↑	Mouse	(Sferruzzi-Perri et al., 2013)
Maternal high fat-low fibre	Placenta	*Igf2*	E13.5	↑	Rat	(Lin et al., 2012)
Maternal low fat	Placenta	*H19*	E13.5 E17.5	↑ ↑	Rat	(Lin et al., 2012)
Maternal low protein	Liver	*Gnas*(LP gestation only)	P21, P84	↓	Mouse	(Ivanova et al., 2012)
	Liver	*Grb10*(LP lactation only)	P21	↑	Mouse	(Ivanova et al., 2012)
	Various brain regions	*Cdkn1c* *Igf2*	Adult Adult	↑ ↑	Mouse	(Vucetic et al., 2010b)

		Tssc4	Adult	↑		
	Blastocyst	*H19*		↓	Rat	(Kwong et al., 2006)
	Liver	*H19* *Igf2*	E20, P0 E20, P0	↓,↑(MO) ↓,↑(MO)	Rat, mouse	(Kwong et al., 2006), (Gong et al., 2010)
Maternal food restriction	Hypothal-amus	*Peg3*	E13	↑	Mouse	(Broad and Keverne, 2011)
	Placenta	*Peg3*	E13, E16.5	↓, ↑	Mouse	(Broad and Keverne, 2011)
	Brain	*Cdkn1c* *Snrpn* Peg3 (protein only)	E16.5 E16.5 E16.5	↓ ↓ ↑	Mouse	(Radford et al., 2012)
	Liver	*H19* *Igf2R* *Zac1* *Grb10* *Peg3*	E16.5 E16.5 E16.5 E16.5 E16.5	↑ ↑ ↑ ↑ ↑	Mouse	(Radford et al., 2012)
Intrauterine growth restriction/Small for gestaional age	Placenta	*PHLDA2* *MEST* *MEG3* *GATM* *GNAS*	Adjusted for gestational age	↑ ↓ ↓ ↓ ↓	Human	(McMinn et al., 2006)

		PLAGL1 *CDKN1C* *IGF2*		↓ ↑ ↓		
	Placenta	*IGF2*	Second trimester	↓	Human	(Cordeiro et al., 2014)
	Fetus	*IGF2*	Second trimester	↓	Human	(Cordeiro et al., 2014)
	Placenta	*PHLDA2*	Neonates >26 weeks	↑	Human	(Guo et al., 2008)
Maternal bisphenol A exposure	Placenta	*Snrpn* *Kcnq1ot1* *Ube3a* *Cdkn1c*	E9.5 E9.5 E9.5 E9.5	↑ ↑ ↓ ↓	Mouse	(Susiarjo et al., 2013)
	Embryo	*Igf2*	E9.5	↑	Mouse	(Susiarjo et al., 2013)
	Brain	*Ube3a*	E12.5	↓	Mouse	(Susiarjo et al., 2013)
Maternal dexam-ethasone treatment	Liver	*Igf2* *Cdkn1c* *Grb10* *H19*	E20 E20 E20 E20	↑ ↑ ↑ ↑	Rat	(Drake et al., 2011)
	Placenta	*Phlda2* *Slc38a4*	E20 E20	↓ ↑	Rat	(Drake et al., 2011)
Low post-natal maternal	Ventral midbrain	*Cdkn1c*	P6	↑	Rat	(Jensen Peña et al., 2014)

licking/ grooming						

1.5 Aims of this work

The aim of this work was to characterise the behavioural and neurochemical consequences of an ectopic increase in *Cdkn1c* expression in a mouse line carrying a BAC transgene spanning *Cdkn1c* (*Cdkn1c*^{BACx1} line) in comparison to wt littermates (John et al., 2001). This work was performed alongside an analysis of a second BAC transgenic line which spanned the same genomic region but with a *β-galactosidase* reporter construct inserted into *Cdkn1c* to disrupt transgenic *Cdkn1c* expression (*Cdkn1c*^{BACLacZ} line). This second line allows attribution of phenotypes specifically to an increased dosage in *Cdkn1c*. *Cdkn1c* was reported to be over expressed from the BAC transgene in *Cdkn1c*^{BACx1} animals at E13.5 in the developing midbrain and anterior pituitary (John et al., 2001). Detailed characterisation of the consequences of *Cdkn1c* over-expression on neural development has not been carried out and this forms the first part of this current study, focussing on the dopaminergic system, given the role of *Cdkn1c* in this system (Joseph et al., 2003; Freed et al., 2008).

The behavioural consequences of increased dosage of *Cdkn1c* have not been examined. Given the documented role for *Cdkn1c* in dopaminergic neurons proliferation and the expression of the transgene in the developing mid brain at E13.5 (John et al., 2001), this study particularly focuses on dopaminergic driven behaviours. Additionally, given the apparent converge of imprinted gene function on social behaviours (Garfield et al., 2011; Jensen Peña et al., 2014; McNamara and Isles, 2014) the consequences for the social environment after *Cdkn1c* overexpression are examined in this study.

Vucetic *et al.* (2010b) had reported the elevated adult neural expression of *Cdkn1c* in offspring exposed *in utero* to a low protein maternal diet (Vucetic et al., 2010b). Given the sensitivity of *Cdkn1c* to the *in utero* and early postnatal environment and its prominent role in neural development, the second part of

this study focused on examining *Cdkn1c* expression and imprinting in response to a variety of maternal conditions, alongside other neurally important, imprinted genes. Finally, to begin to tie together in utero exposures with *Cdkn1c* over expression, this work aimed to examine the consequences on the dopaminergic system after gestational adversity in the context of findings from the transgenic model.

Specifically the aims of this thesis were to:

1. Characterise the neurochemistry of the adult male *Cdkn1c*^{BACx1} line and the *Cdkn1c*^{BACLacZ} line, focusing on the dopaminergic system, basally and in response to external stimuli (Chapter 3).
2. Perform basic behavioural characterisation of the adult male *Cdkn1c*^{BACx1} line and the *Cdkn1c*^{BACLacZ} line to asses motor, anxiogenic, sensorimotor gating and response to a dopaminergic system agonist (Chapter 4).
3. Examine the effects of an increased dosage of *Cdkn1c* on motivational and hedonic responding (Chapter 5).
4. Determine the consequences of increased *Cdkn1c* expression for a social group of *Cdkn1c*^{BACx1} animals and wt cage mates in comparison to social groups of *Cdkn1c*^{BACLacZ} animals and wt cage mates (Chapter 6).
5. Evaluate the sensitivity/resilience of imprinted gene expression in the developing fore- and mid- brain to a suboptimal prenatal diet and assess parental allele contribution to any alterations in *Cdkn1c* (Chapter 7).
6. Assess the consequences for the dopaminergic system of altered imprinted gene dosage following prenatal dietary alterations.

Chapter 2: Methods

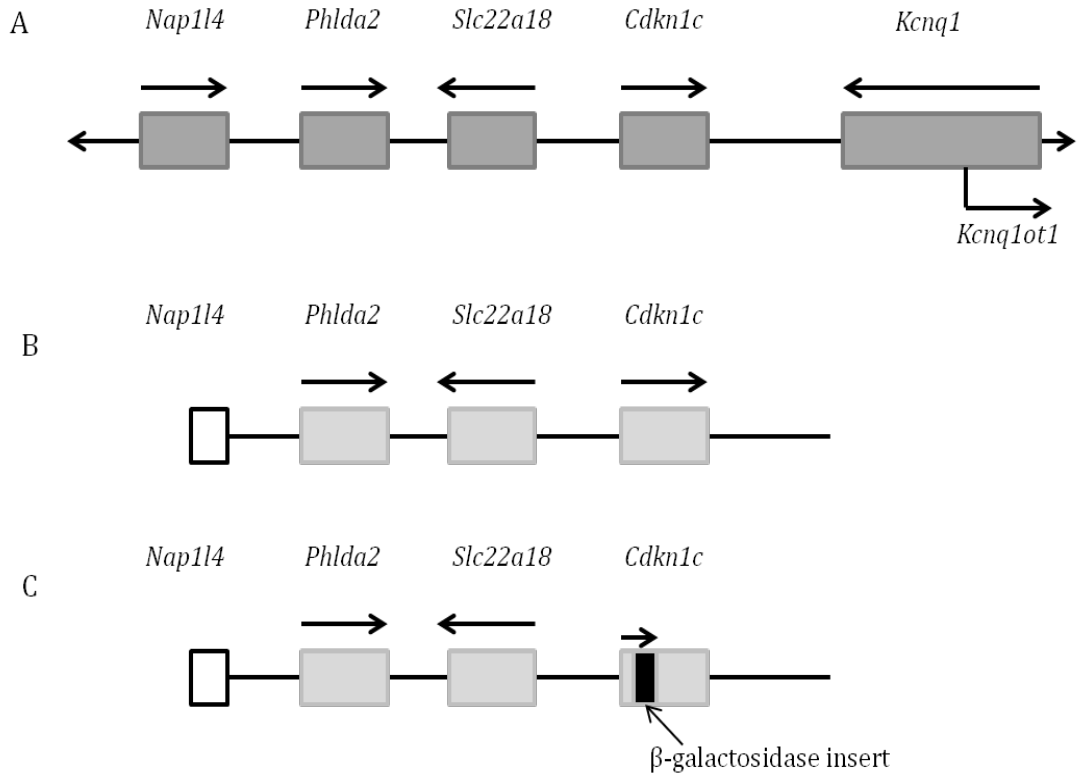
2.1 Animals

2.1.1 Animal lines

2.1.1.1 *Cdkn1c*^{BACx1} and *Cdkn1c*^{BACLacZ}

Two transgenic mouse lines were used for experiments to study the effects of *Cdkn1c* over-expression. The experimental line *Cdkn1c*^{BACx1} possesses one copy of a BAC (Genome Systems 144D14) that spans the *Cdkn1c* gene and two other genes, *Phlda2* and *Slc22a18*. This BAC is illustrated in Figure 2.1B. This line were initially generated by electroporating the linearised BAC in mouse 129 RI ES cells, making chimeras and then identifying transgenic offspring (John et al., 2001). The line was initially bred into an MF1 background as no survivors were found on a pure 129 background. For the purposes of this study, the lines were bred for ≥8 generations in the BL6 background. The reporter line *Cdkn1c*^{BACLacZ} possesses a modified version of BAC 144D14, illustrated below in Figure 2.1C. For creation of this reporter line, the BAC 144D14 clone was modified by homologous recombination of a *β-galactosidase* reporter construct (*p57Kip2-IRESβgeoloxPalkP*) into the *Cdkn1c* locus. Transgenic mice were generated by pronuclear injection of the construct into F_1 C57BL/6 x CBA embryos. The line can be used as a reporter (LacZ staining) to observe *Cdkn1c* expression originating from the transgene, as has been performed previously (John et al., 2001). This line also acted as a control for the experimental line to attribute any phenotypes observed specifically to the over expression of *Cdkn1c* (i.e. present in line 1 *Cdkn1c*^{BACx1}, absent in line *Cdkn1c*^{BACLacZ}). Trangenic animals were generated by mating male transgenics with wt females, all on a C57BL/6 background.

Figure 2.1: *Cdkn1c* containing locus and position of transgenes. A) Representation of five of the imprinted genes found in mouse distal chromosome 7. The five pictured genes are maternally expressed imprinted genes, while the non-coding *Kcnp1ot1* transcript is paternally expressed. B) Representation of the unmodified BAC144D14. Line *Cdkn1c*^{BACx1} contains one copy of the BAC and all three genes are expressed from the transgenic at level similar to the endogenous loci (Andrews et al., 2007; Tunster et al., 2010). C) Representation of modified BAC transgene. Line *Cdkn1c*^{BACLacZ} carries one copy of this modified BAC in which a *β-galactosidase* insert interrupts expression of *Cdkn1c* expression from the BAC. *Phlda2* and *Slc22a18* expression are the only genes expressed from this modified BAC (Andrews et al., 2007; Tunster et al., 2010).

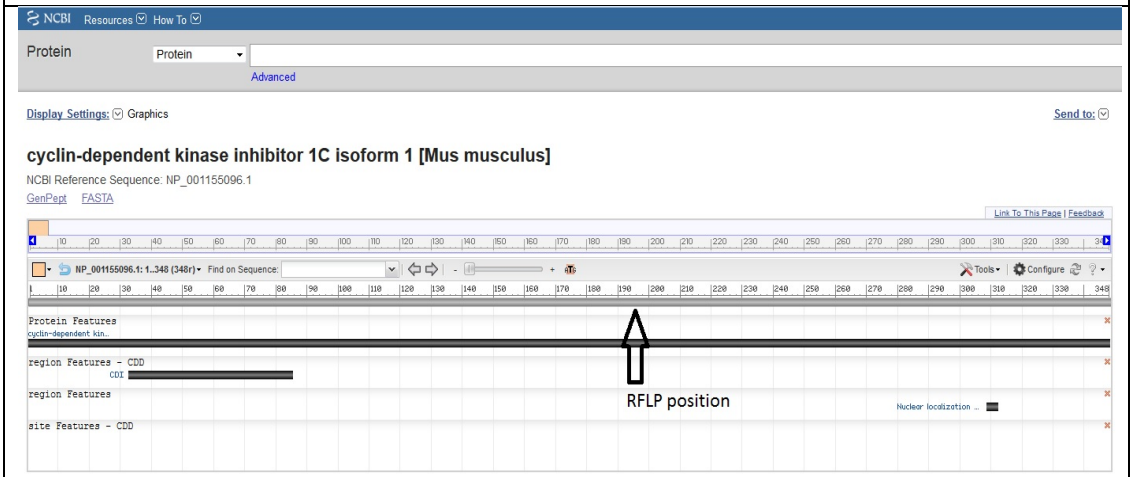


2.1.1.2 *Cdkn1c* RFLP line

For analysis of parent of origin expression of imprinted genes located on distal chromosome 7, a wt line was used, which was polymorphic at this site. This line was generated by mating an individual from the C57BL/6 strain with an animal from the genomically polymorphic out-bred mouse strain, *Mus spretus*.

Offspring were back crossed onto a B57BL/6 background for >8 generations, selecting for a restriction fragment length polymorphism (RFLP) in *Cdkn1c*, position of RFLP is demonstrated in Figure 2.2. Material for parent of origin analysis was generated by mating an RFLP carrying male with a C57BL/6 female.

Figure 2.2: Position of RFLP within the *Cdkn1c* protein



2.1.2 Animal husbandry

Mice were housed in cages in cohorts not greater than five individuals per cage in non-barrier conditions as per Home Office regulations. Environment was kept at 20°C ± 1°C on a 12 hour light-dark cycle with standard chow (Harlan, Oxfordshire) and water freely accessible, except where described in experimental chapters. Mice were weaned at four weeks and caged separately from parents in single sex cages. For generation of material from embryonic time points presence or absence of a vaginal plug was examined each morning before 10:00. Females were removed from sire's cage each morning and replaced each night to minimise number of missed plugs.

For water deprivation, water access was limited initially to 4 hours per day and water bottles were weighed before and after access to ensure consumption. Animal weight was monitored to ensure weight gain over time. When consumption during 4 hours access had stabilised, access was reduced to 2 hours per day. Water bottles were weighed before and after access to ensure

consumption and animal weight was monitored daily. For testing water access was provided for 2 hours immediately following testing.

For food deprivation, food was removed from hopper 16 hours prior to testing and replaced immediately following testing. Animal weight was monitored daily to ensure weight gain.

2.1.3 Genotyping, RFLP detection and sex-typing

Ear biopsy from 4 week old animals was digested overnight in 200 µl lysis buffer (0.1 M Tris.HCl pH 8.5, 0.005 M EDTA pH8.0, 0.02% SDS, 0.2 M NaCl, 100 µg/ml Proteinase K (Promega)) at 55-60°C. Embryonic yolk sacs were treated in the same manner but lysed in 300 µl of lysis buffer. The following morning, samples were vortexed briefly and centrifuged at 13000 rpm for 14 minutes to pellet cellular debris. Supernatant was then transferred to a fresh 1.5 ml tube and 1X volume of isopropanol was added to precipitate gDNA. Samples were mixed thoroughly and incubated at 4°C for 30 minutes to promote gDNA precipitation. Following incubation, samples were vortexed briefly and centrifuged at 13000 rpm for 12 minutes to pellet gDNA. Supernatant was removed and pellet was allowed to air dry at room temperature for 10 minutes. Pellet was resuspended in 30 µl (Ear biopsy) or 80 µl (yolk sac) TE buffer (10mM Tris pH8.0, 1mM EDTA pH8.0). 1 µl of this was used as template for PCR. PCR mastermix is shown in table 2.1.

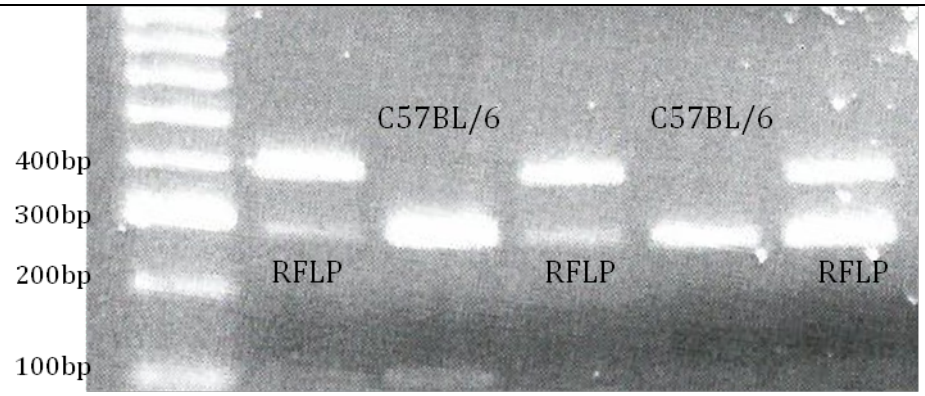
Amplification of target was carried out under the following conditions:
For *Cdkn1c*^{BACx1} and *Cdkn1c*^{BACLacZ} :1. 94°C 15 minutes, 2. 94°C 30 secs, 3. 58.9°C 30 secs, 4. 72°C 30 secs, repeat cycle 2-5 35 times, 6. 72°C 5 minutes.
For RFLP detection: 1. 94°C 15 minutes, 2. 94°C 30 secs, 3. 64.5°C 30 secs, 4. 72°C 30 secs, repeat cycle 2-5 35 times, 6. 72°C 5 minutes.
For sex typing: 1. 94°C 15 minutes, 2. 94°C 45 secs, 3. 61°C 45 secs, 4. 72°C 45 secs, repeat cycle 2-5 35 times, 6. 72°C 5 minutes.

For *Cdkn1c*^{BACx1} and *Cdkn1c*^{BACLacZ} genotyping and sex-typing PCR products were separated on a 1% agarose gel and viewed under U.V light. A negative and a positive control were used for every reaction. For sex-typing an autosomal linked gene (*Om1a*) and a Y linked gene (*Ssty*) were amplified, females were identified by a single band and males with a double band.

For RFLP detection, following PCR amplification, PCR product was precipitated using 0.3 M sodium acetate pH, 2.5X 100% ethanol and 1 µl glycogen. Mixture was incubated at -20°C for 30 minutes to enhance PCR product precipitation. Following incubation, samples were vortexed briefly and centrifuged at 13000 rpm for 12 minutes to pellet PCR product. Supernatant was digarded and pellet was immediately resuspended in 20 µl digestion mix (1X restriction buffer 4 (New England Biolabs, UK), 1 ul *AvaI* (New England Biolabs, UK), 18 µl nuclease free water (Life Technologies, UK). Samples were incubated at 37°C for at least two hours to ensure complete digection. Samples were separated on a 1% agarose gel and viewed under U.V light. PCR amplified a 386 bp region containing an RFLP site. *AvaI* recognises CCCGAG (CYCGRG) on the C57BL/6 allele and cuts it, resulting in two fragments, 281 and 105 bp in length. The corresponding *M. Spretus* site has a polymorphism CCTGAG that the restriction enzyme does not recognise. Therefore, presence of the RFLP is indicated by 386 (undigested), 281 and 105 (digested) bp bands. Absence of an RFLP (two 100% C57BL/6 chromosomes) is indicated by presence of 281 and 105 (digested) bp bands. The 105 bp band is not always visible after resolving. A representative image is shown in Figure 2.3.

Table 2.1 1X mastermix for PCR	
Reagent	**Volume (per reaction, µl)**
10X PCR Buffer with 15 mM $MgCl_2$ (Qiagen)	2.5
$MgCl_2$ (25 mM)	2
dNTPs (4 mM)	2
Primers F + R (25 µM of each)	1
HotStart Taq (Qiagen)	0.25
Template	1
ddH_20	to 25 µl

Figure 2.3: A representative image of genotyping for presence of RFLP. All animals were either homozygous for C57BL/6 alleles or heterozygous.



2.1.4 Serum collection

For serum collection animals were weighed and injected intraperitonally (i.p.) with a terminal dose of sodium pentobarbinal (100 mg/kg; Euthatal; Merial Animal Health, Harlow, UK). Once animal was unresponsive to limb and tail stimulation the abdominal cavity and heart was exposed. A 19G needle attached to a 1 ml syringe was used to pierce the left ventricle and the syringe was held at negative pressure to draw blood out. Collected blood was allowed to coagulate for 30 minutes in microtainer tubes (BD, Oxford, UK) at room temperature (RT). Samples were centrifuged at 13,000 rpm for 5 minutes and snap frozen for later analysis.

2.1.5 Dissections

Following serum collection, animals were decapitated and the brain was removed from the skull. The following regions were then isolated: hypothalamus, frontal cortex, dorsal and ventral striatum. Hypothalamus removed initially from the ventral surface of the brain. The brain was then turned dorsal side upwards and the frontal cortex was isolated in the coronal plane. This was achieved by removal of the olfactory bulb and making a cut at a 45° angle from 2 mm caudal to the front of the brain. 1 mm caudally from this cut site a second cut was made at a 90° angle, the tissue isolated here was discarded. A further 2 mm caudally a second cut was made at a 90° angle. The tissue isolated here constituted the majority of the striatum, plus surrounding cortices. The striatum was isolated by removal of the surrounding tissue and then was subdivided into dorsal and ventral striatum. Each tissue piece was snap frozen and stored for later analysis.

2.2 qPCR

2.2.1 RNA extraction

Three methods of RNA extraction were used in this work. For Chapter 3.3.1, Figure 3.1A, RNA was extracted and DNase treated by G.I. McNamara and Dr. D Relkovic. All RNA to cDNA conversion and qPCR was carried out by G.I. McNamara.

2.2.1.1 Chapter 3, embryonic brain *Cdkn1c*^{BACx1}

RNA and protein were extracted simultaneously using PARIS kit (Life Technologies, UK) according to manufactures instructors. Samples were homogenised using 300 µl cell disruption buffer and RNase free pellet pestle (Anachem, UK). RNA was stored at -80°C.

2.2.1.2 Chapter 3, wt embryonic tissue and adult neural tissue

400 µl trizol was added to each sample while frozen. Homogenisation was carried out using RNase free pellet pestle (Anachem, UK) until there were no visible particles. Samples were centrifuged at 4°C at 13000g for 10 minutes. Supernatant was transferred to a fresh RNase free 1.5 ml tube and 200 µl

chloroform (Sigma-Aldrich, UK) was added. Samples were vortexed for 15 s and incubated at room temperature for 10 minutes. Samples were centrifuged at 4°C at 13000g for 10 minutes. Aqueous phase was transferred to a fresh RNase free 1.5 ml tube and 300 µl isopropanol (Thermo-Fisher Scientific, UK) was added. Samples were vortexed for 15 s and incubated at room temperature for 10 minutes. Samples were centrifuged at 4°C at 13000g for 10 minutes and a pellet was visible. Supernatant was removed and sample was washed in 500 µl 75% ethanol (Thermo-Fisher Scientific, UK). Samples were centrifuged at 4°C at 13000g for 5 minutes and supernatant was removed. Pellet was air dried for 15 minutes at room temperature and resuspended in 30 µl nuclease free water (Life Technologies, UK) and stored at -80°C.

2.2.1.3 Chapter 7, embryonic brain and kidney

RNA was extracted using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, UK) according to manufactures instructors. Samples were homogenised using 200 µl buffer and MP lysing matrix D tubes (Anachem, UK) and fast prep 120 (Thermo-Fisher Scientific, UK). Homogenisation was performed twice for 10 s at 5 m/s. RNA was stored at -80°C.

2.2.2 DNase treatment

All RNA was DNase treated following extraction. Up to 10 µg of RNA was treated using DNA-free DNase treatment and removal kit (Applied Biosystems, UK) according to the manufacturer's instructions.

2.2.3 RNA to cDNA conversion

Two methods of reverse transcription were used in this work.

2.2.3.1 Chapter 3

Equal amounts of RNA, up to 5 µg were reverse transcribed using RNA to cDNA EcoDry (random hexamers) premix strips (Takara Bio Europe, France). cDNA was diluted 1:50 and stored at -20°C.

2.2.3.2 Chapter 7

Equal amounts of RNA, up to 5 µg were reverse transcribed using the mastermix below (Table 2.3). RNA was incubated with random hexamer primers for 10 minutes at 70°C to allow annealing. Remainder of master mix was added and

samples (RT+) were incubated at 37°C for 1 hour. A negative control (RT-)was included in which no enzyme was added. Reaction was stopped by sample incubation at 70°C for 15 minutes. cDNA was diluted 1:50 in TE buffer and stored at -20°C. cDNA was checked by performing a PCR on RT+ and RT- samples using the mastermix outlined above (Table 2.1) with primers for *βactin*. PCR products were resolved on a 1% agarose gel under UV light.

Table 2.2: 1X master mix for RNA reverse transcription.

Reagent	**Volume per reaction (µl)**
5X first strand sunthesis buffer (Promega, UK)	4
dNTPs (10 mM)	1
Random hexamers (0.5 µg/ml) (Promega, UK)	1
MMULV reverse transcriptase (Promega, UK)	1
DNase treated RNA	Up to 5 µg
Nuclease free H_20	Up to 20 µl

2.2.4 qPCR protocol

All qPCR primers were designed to work optimally with an annealing temperature of 60 °C, spanned at least one intron and the product size was less than 200 bp. Primer information detailed in Table 2.6. For every experiment amplification of all genes of interest plus two housekeeping genes was performed in triplicate in the same qPCR run. 25 ng of cDNA was used for each reaction. The mastermix is shown in Table 2.3. The cycling conditions used for all qPCR reaction was: 1. 94°C 10 minutes, 2. 94°C 20 secs, 3. 60°C 20 secs, 4. 72°C 20 secs, repeat cycle 2-4 45 times. Fluorescence of each reaction was acquired at step 4 for each cycle. Following this thermo-cycling step the PCR product was heated in 1°C steps from 50- 99 °C, held for 2s at each step and fluorescence was recorded. This allowed quality control of qPCR product.

2.2.5 High resolution melt analysis (HRM) protocol

HRM analysis was carried out as described in Chapter 7.2.4 using the master mix shown below in table 2.4

Table 2.3: 1X qPCR master mix	
Reagent	**Volume per reaction (µl)**
2X SensiMix SYBR No-ROX (Bioline, UK)	10
Forward primer	1.4
Reverse primer	1.4
cDNA template	50 ng
Nuclease free water	To 20 µl

Table 2.4: 1X HRM master mix	
Reagent	**Volume per reaction (µl)**
2X SensiMix HRM (Bioline, UK)	10
EvaGreen dye	1
Forward primer	1.4
Reverse primer	1.4
cDNA template	50 ng
Nuclease free water	To 20 µl

2.3 Western blotting

2.3.1 Protein extraction

Tissue samples were homogenised using using RNase free pellet pestle (Anachem, UK) in 200 µl filter sterilised treatment buffer (Table 2.5) without 2-mercaptoethanol until there were no visible particles. Samples were centrifuged briefly to pellet lysate and then sonicated briefly to shear DNA and reduce viscosity of sample. Sample was the centrifuged for at 13000 g for 10 minutes at 4°C. BCA assay was then carried out to quantify protein in sample.

2.3.2 BCA assay

BCA assay was carried out using BCA Protein Assay Kit (Thermo-Fisher Scientific, UK) according to manufacturer's instructions. Briefly, a range of known concentration protein standards were prepared from a stock bovine serum albumin standard (Thermo-Fisher Scientific, UK), ranging from 25-2000 µg/ml. Unknown protein samples to be quantified were diluted 1:10. 50 µl of known standards and unknown samples were transferred to a 96 well plate (Thermo-Fisher Scientific, UK) and 200 µl of a 50:1 reagent A: reagent B mixture was added. Each sample was quantified in duplicate. Samples were incubated at 37 °C for 30 minutes and following this absorbance was read at 562 nm using a microplate spectrophotometer (µQuant, BioTek, UK). Unknown protein samples were quantified by comparing to known standard absorption at 562 nm. Unknown samples concentration was diluted to 5 mg/ml using treatment buffer including 5% 2- mercaptoethanol (Sigma-Aldrich, UK) and bromophenol blue (Sigma-Aldrich, UK).

Component	**Stock**	**Volume/Amount**
75 mM Tris HCL pH6.8	1.5M	0.5 ml
3.8% SDS(Sigma-Aldrich, UK)	20%	1.9 ml
4M Urea (Sigma-Aldrich, UK)	8M	5.0 ml
20% glycerol (Thermo-Fisher Scientific, UK)	100%	2.0 ml
dH_2O		Up to 10 ml
5% 2-mercaptoethanol	100%	0.5 ml
Bromophenol blue		0.2 g

Table 2.5: Treatment buffer for protein extraction

2.3.3 Gel electrophoresis and Western blot

Samples were defrosted on ice and 20 µl (100 µg) was transferred to a fresh tube with 1 µl 2-mercaptoethanol to ensure protein denaturation. 75 µg of protein was analysed for each sample. Proteins were electrophoretically separated on a 10% bis-tris acrylamide gel (NuPAGE Novex, Life Technologies,

UK) at 200V for 35 mins using 1X NuPAGE MES SDS Running Buffer (Life Technologies, UK). Proteins were then transferred onto a nitrocellulose membrane (pore size 0.45 μm, Life Technologies, UK) at 400 mA for 2 hours at room temperature in tris-glycine methanol transfer buffer (10% (v/v) methanol (Thermo-Fisher Scientific, UK), 0.02 M tris (Thermo-Fisher Scientific, UK), 0.19 M glycine (Sigma-Aldrich, UK)). Membranes were subsequently blocked in 1% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, UK) tris-buffered saline (TBS) for 1 hour at room temperature with agitation. Primary antibodies were diluted in 1% (w/v) BSA TBT-T (1% (v/v) Tween-40 (Sigma-Aldrich, UK) TBS) and incubated with membrane overnight at 4°C with mild agitation. Antibodies used were rabbit polyclonal anti-Th (Abcam, UK) diluted in 1:1000 and mouse monoclonal anti-βactin (Sigma-Aldrich, UK) diluted 1:3300. Membranes were washed in TBS-T for 10 minutes at room temperature four times. Secondary antibodies used were either conjugated to an Alexa Fluor 680 dye (Life Technologies, UK), for visualisation of test protein or were conjugated to IRDye 800 (LI-COR, UK) for visualisation of βactin loading control. Secondary antibodies diluted 1:3000 in 1% BSA TBS-T and membranes were incubated in this at room temperature for one hour with agitation. Membranes were washed in TBS-T for 10 minutes four times and once in PBS for 5 minutes at room temperature. Proteins were visualised using LI-COR Odyssey infrared imaging system (LI-COR, UK).

2.3.4 Relative protein quantification

Relative protein abundance was quantified using a computerized image-analysis system (NIH ImageJ software version 1.45s; http://rsb. info.nih.gov/ij/). Protein band was outlined and average intensity was recorded. This was performed for Th and βactin bands and relative Th abundance was calculated using the formula (average intensity of Th band)/(average intensity for βactin band). Statistical analysis was carried out on this value.

2.4 Immunohistochemistry and image analysis

2.4.1 Immunohistochemistry

Perfusion of the brain and sectioning was carried out as described in this thesis chapter 3.2.1.3. Coronal sections stored in cyroprotectant were washed four times for 10 minutes at room temperature with mild agitation in TBS (0.1 M tris 0.15 M NaCl (Thermo-Fisher Scientific, UK) pH 7.4). To block endogenous peroxidises, sections were incubated in 0.6% (v/v) hydrogen peroxidise in TBS at room temperature for 30 minutes. This was followed by three 10 minute washes in TBS. Sections were blocked in 3% (v/v) normal goat serum (NGS) TBS-T (0.1% Triton-X (Sigma-Aldrich, UK) TBS). Primary antibody was diluted in 3 % NGS TBS-T and sections were incubated in this overnight at 4°C. Antibodies used were rabbit polyclonal anti-NeuN (1:1000) (Abcam, UK) and rabbit polyclonal anti-Th (1:1000) (Abcam, UK). Following primary incubation sections were washed three times for 10 minutes in TBS-T. The VECTASTAIN ABC kit (Vector Labs, Peterborough, UK) was used as per manufacturer's instructions. Briefly, biotinylated secondary antibody was diluted 1:200 in 3% NGS TBS-T and sections were incubated in this for one hour at room temperature with mild agitation. This was followed by another wash step as before. Sections were then incubated in avidin- biotinylated horseradish peroxidise (HRP) reagent for one hour at room temperature with mild agitation. This was followed by a wash step as above, plus two additional washes in 0.05 M Tris buffer. The DAB peroxidise substrate kit (Vector Labs, UK) was used as per manufacturer's instructions to detect biotinylated HRP bound to secondary antibody. Sections were washed in ice cold PBS to stop the reaction, followed by a final TBS-T wash. Sections were mounted on gelatin coated slides (Thermo-Fisher Scientific, UK) and allowed to dry overnight at room temperature. Sections were dehydrated by a series of 2 minute incubations in 50%, 70%, 90%, 100% and 100% ethanol, followed by two serial incubations in xylene (Thermo-Fisher Scientific, UK) and a cover slip was affixed using DPX (VWR, UK) as a mountant. Slides were dried overnight at RT.

2.4.2 Image analysis

Images were acquired at 1.5X (for Th immuno-reactivity) or 5X (for NeuN immunoreactivity) magnification using Leica Olympus DP73. For Th immuno-reactivity all sections containing the straitum were imaged. For NeuN 10 sections distributed evenly throughout the striatum were chosen for analysis. A single image was taken of the striatum and another of the adjacent cortex, for each hemisphere, a total of 20 images per animal. All image analysis was carried out using ImageJ. For striatal Th average optical density of the striatum, separated into dorsal and ventral regions at the as outlined in Figure 2.4, was obtained and the average background staining from the cortex was subtracted to give a value comparable between sections. This measurement also allowed analysis of striatal area. Cell number was calculated using ImageJ cell counter plugin (NIH ImageJ software version 1.45s; http://rsb. info.nih.gov/ij/). Briefly the image was converted into an 8-bit black and white image and cell counting was performed. This was performed for all striatal and cortical sections to obtain an estimate of cell number. Representatives of image processing steps are outlined in Figure 2.5A-H.

Figure 2.4: 1.5X magnification of a representative section stained for Th. Regions identified as dorsal and ventral striatum during analysis are outlined.

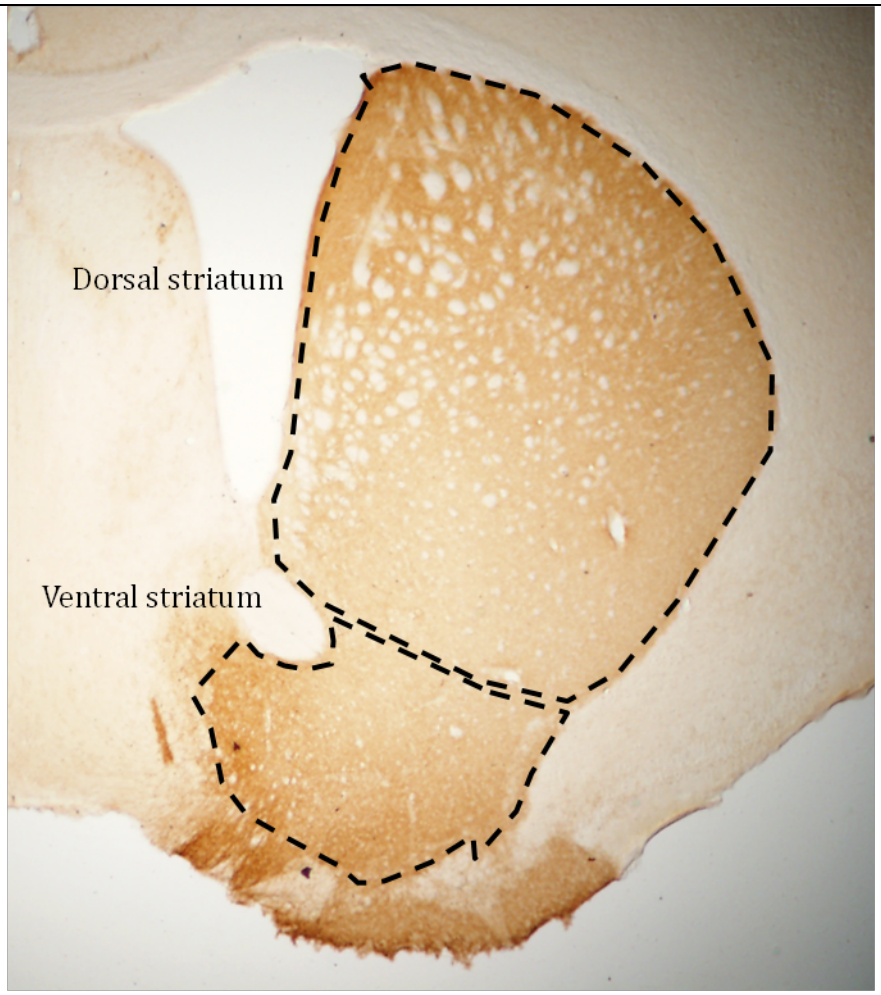


Figure 2.5: 5X magnification of a representative section stained for NeuN. Image processing stages are out lined below. A) Captured image. B) region of interest (cortex in this image) was selected and surround was cleared. C) Image was converted into 8-bit greyscale image. D) Background was subtracted using 6 pixel rolling ball radium criteria. E) Image was auto-thresholded such that each pixel because binary. F) Image was auto-eroded and dilated to remove small, non cellular pixels. G) Watershed analysis was performed to auto-detect individual objects. H) Auto particle analysis was carried out

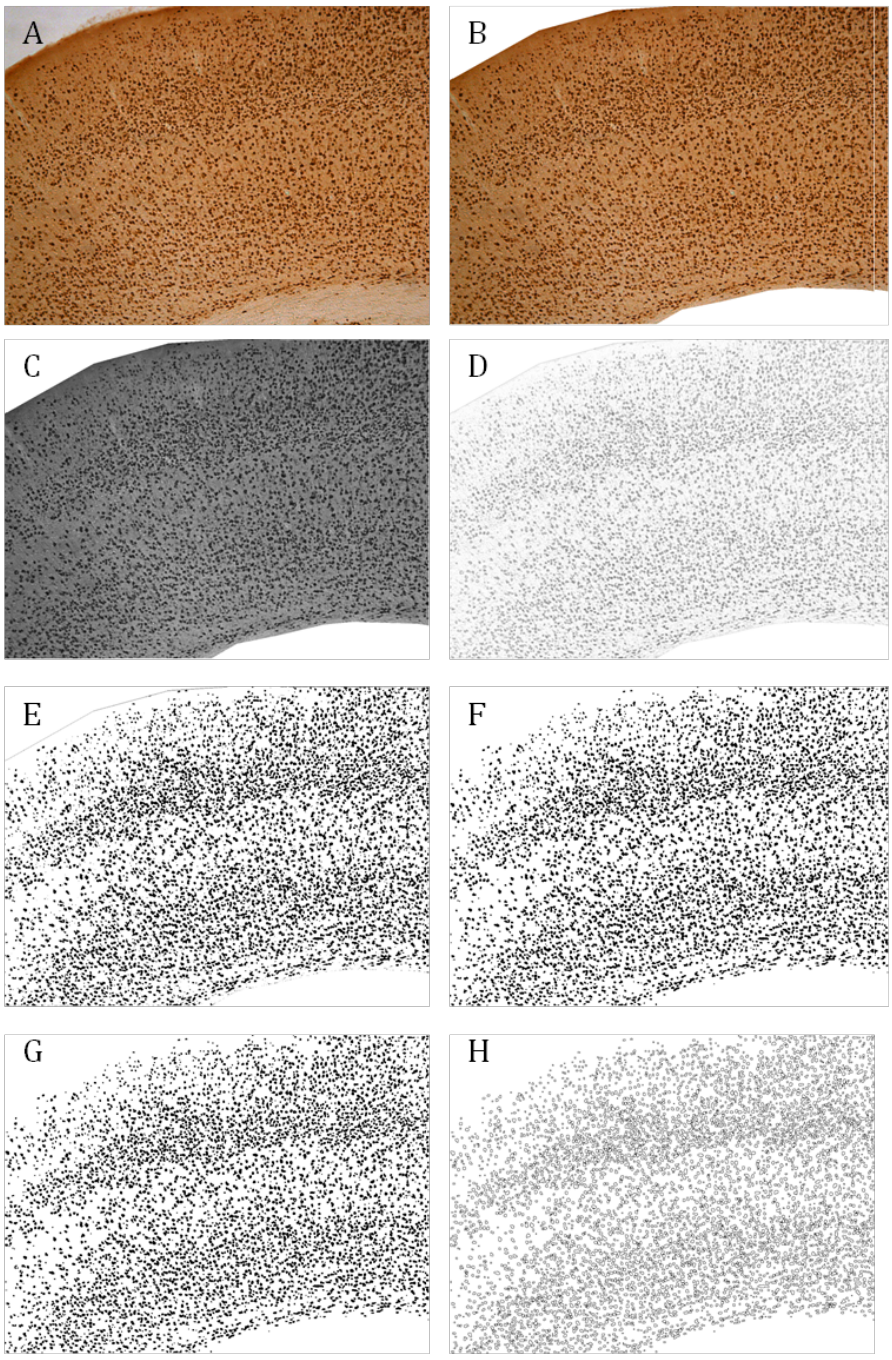


Table 2.6: List of primers used in this thesis with accompanying sequences and annealing temperatures				
Primer name (target)	Sequence 5'-3'	PCR/ qPCR	Annealing temp. (°C)	Product size (bp)
R85 (BAC transgene)	GGGCACCAATAACTGCCTTA	PCR	58.9	575
R86 (BAC transgene)	GCGTGTTACGGTGAAAACCT	PCR	58.9	
R299 (RFLP site)	GGCTTCAGATCTGACCTCAG	PCR	64.5	386
R300 (RFLP site)	AGAGAGGCTGGTCCTTCAGC	PCR	64.5	
Cdkn1c-F (*Cdkn1c)*	AGAGAACTGCGCAGGAGAAC	qPCR	60	141
Cdkn1c-R (*Cdkn1c*)	TCTGGCCGTTAGCCTCTAAA	qPCR	60	
Dat-F (*Dat*)	GGTGCTGATTGCCTTCTCCA	qPCR	60	116
Dat-R (*Dat*)	AGAAGACAACGAAGCCAGAGGAG	qPCR	60	
Drd1-F (*Drd1*)	GGACACCGAGGATGACAACT	qPCR	60	89
Drd1-R (*Drd1*)	TGGCTACGGGGATGTAAAAG	qPCR	60	
Drd2-F (*Drd2*)	CTGGTGTGCATGGCTGTATC	qPCR	60	119
Drd2-R (*Drd2*)	TAGACGACCCAGGGCATAAC	qPCR	60	
Dlk1-F (*Dlk1*)	GAAAGGACTGCCAGCACAA	qPCR	60	141

Dlk1-R (*Dlk1*)	CACAGAAGTTGCCTGAGAA	qPCR	60	
Grb10-F (*Grb10*)	TGCACCACTTCTTGAGGATG	qPCR	60	202
Grb10-R (*Grb10*)	ACCAGTGAGCTCCGGAAATG	qPCR	60	
Igf2-F (*Igf2*)	GTCGATGTTGGTGCTTCTCA	qPCR	60	195
Igf2-R (*Igf2*)	AAGCAGCACTCTTCCACGAT	qPCR	60	
Nnat-F (*Nnat*)	AGAAAAGCAGCACCGACAAT	qPCR	60	204
Nnat-R (*Nnat*)	GGCTGTTCGATCTTCATGGT	qPCR	60	
Nurr1-F (*Nurr1*)	CGGACTGCAGGACGAGC	qPCR	60	115
Nurr1-R (*Nurr1*)	GCTATTGGGAATCCAGCCCG	qPCR	60	
Om1a-F (*Om1a*)	TTACGTCCATCGTGGACAGCAT	PCR	61	245
Om1a-R (*Om1a*)	TGGGCTGGGTGTTAGTCTTAT	PCR	61	
Peg3-F (*Peg3*)	AAAACTCACCACTCCGTTGG	qPCR	60	190
Peg3-R (*Peg3*)	GTCTCGAGGCTCCACATCTC	qPCR	60	
Sno116-F (*Sno116*)	ATCTAATGATTCCCAGTCAAACAT	qPCR	60	53
Sno116-R (*Sno116*)	TCACTCATTTTGTTCAGCTTTTCC	qPCR	60	
Ssty-F (*Ssty*)	CTGGAGCTCTACAGTGATGA	PCR	61	343

Ssty-R (*Ssty*)	CAGTTACCAATCAACACATCAC	PCR	61	
Th-F (*Th*)	AATTCCCCACGTGGAATACA	qPCR	60	83
Th-R (*Th*)	GGGTAGCATAGAGGCCCTTC	qPCR	60	
Hprt-F (*Hprt*)	ATGATCAGTCAACGGGGGA	qPCR	60	189
Hprt-R (*Hprt*)	GAGAGGTCCTTTTCACCAG	qPCR	60	
βactin-F (*βactin*)	TCTGTGTGGATTGGTGGCTCTA	qPCR/ PCR	60	69
βactin-R(*βactin*)	CTGCTTGCTGATCCACATCTG	qPCR/ PCR	60	

Chapter 3: Molecular characterisation of *Cdkn1c*^{BACx1}

3.1 Introduction

During embryonic development *Cdkn1c* has a number of different roles in the nervous system including facets of cell proliferation, differentiation and migration, as detailed in Chapter 1. However, a comprehensive characterisation of the consequences of altered *Cdkn1c* dosage on embryonic and adult neurochemistry has not been carried out. This chapter addresses the developmental and neurochemical consequences of altering the dosage of *Cdkn1c* using two BAC transgenic lines. Animals carrying an extra copy of *Cdkn1c* on a BAC transgene (originally called line 5D3 and renamed *Cdkn1c*^{BACx1}) were previously shown to have approximately a 1.9 fold increase in *Cdkn1c* mRNA levels in the embryonic head at E12.5 but not in the body, where there is minimal transgene expression (Andrews et al., 2007). This was not the case for a line carrying the same genomic region on a BAC in which a β-galactosidase-neomycin fusion gene had been inserted into *Cdkn1c*. This line, called Cdkn1c^{BACLacZ}, reports *Cdkn1c* expression from the transgene but *Cdkn1c* expression is not elevated. A comparison between these two lines allows phenotypic assignment to elevated *Cdkn1c* expression.

Given the established role of *Cdkn1c* in promoting proliferation of dopaminergic neurons in the developing midbrain through cooperation with *Nurr1* (Joseph et al., 2003) and *Cdkn1c* expression in human embryonic stem cells differentiated into Th positive dopaminergic neurons *in vitro* (Freed et al., 2008), the dopaminergic system was examined first. Development of the dopaminergic system in animals over expressing *Cdkn1c* was examined using qPCR from E13.5 to E18.5 to assess the expression levels of markers of dopamine development, *Nurr1* and *Th* (Zetterström et al., 1996; Zetterström et al., 1997). Basal dopaminergic state of the adult male brain was assessed using a combination of qPCR, HPLC and immunohistochemistry to obtain convergent evidence for alterations in the dopaminergic system. Additionally, reactivity and sensitivity of the dopaminergic system was probed by exposing animals to a sub-stimulatory dose of the indirect dopamine agonist, amphetamine. Amphetamine

causes a rapid release of dopamine in the nucleus accumbens (Siciliano et al., 2014). The abundance of cells activated by dopamine binding to its post-synaptic receptors can be indirectly assessed using the immediate early gene, *cfos,* as a marker of cellular activity (Swaney et al., 2007; Swaney et al., 2008; Beiderbeck et al., 2012; Regier et al., 2012; Segovia et al., 2012; Sonntag et al., 2014). This method allows an approximation of the number of dopaminergic innervations and strength of connections in relevant regions. In this case, the focus was on the ventral striatum, the site of the nucleus accumbens.

3.2 Methods

3.2.1 Animals and embryonic dissections

General housing and husbandry conditions were as described in Chapter 2.1.2. For embryonic dissections, pregnant dams were culled by cervical dislocation at the required E after observation of the presence of a vaginal plug. The abdomen was sterilised using 70% ethanol and the uterus was exposed. The uterine horn was rapidly dissection out and placed into ice cold sterile PBS. Fetuses were removed from the yolk sac into ice cold PBS on ice and yolk sac was retained for genotyping. Embryos were decapitated and the whole brain was rapidly isolated and snap-frozen on dry ice in a 1.5 ml centrifuge tube. For E11.5 whole head was taken, for all other timepoints embryonic brain was isolated. Tissue was stored at -80°C until analysis. For qPCR analysis samples were taken from two litters and balanced for litter, sex and genotype. Adult neural tissue was collected from cohorts at the end of behavioural testing as described in Chapter 2.1.5.

3.2.1.1 Injections

Animals were injected intra-peritoneally (i.p.) with saline or 0.5 mg/kg D-amphetamine sulphate (Tocris Bioscience, Bristol, UK) to detect any enhanced sensitivity to amphetamine. Animals were culled 60 minutes after injection by cervical dislocation and dissection of neural tissue was carried out as described in Chapter 2.1.5.

3.2.1.2 Perfusion

Animals were given a terminal dose of sodium pentobarbital solution (100 mg/kg, i.p.; Euthatal; Merial Animal Health, Harlow, UK). Once the animal was unresponsive to paw and tail pinch, the abdominal cavity and heart was exposed. At this point serum was collected at described in Chapter 2.1.4. For perfusion, the right atrium was severed to allow outflow and 25 ml of sterile PBS was perfused through the circulatory system via the left ventricle. Following this 25 ml of 10% neutral buffered formalin solution (Sigma Aldrich, Dorset, UK) was perfused through the circulatory system. Once perfusion was complete the brain was extracted and post-fixed in 10 ml 10% neutral buffered formalin solution overnight at 4°C. Following this, brains were transferred to 30% (w/v) sucrose PBS solution to equilibrate at 4°C. Once equilibration was complete, as indicated by sinking, brains were transferred to fresh 30% sucrose PBS solution and stored at 4°C until processing.

3.2.1.3 Brain sectioning

Brain sectioning was carried out on a sliding freezing microtome. After mounting on the platform, brains were frozen and 40 µm thick coronal sections were obtained. Slices were retained from the frontal cortex (most anterior) until the corpus callosum was observed to separate between the two hemispheres. Slices were stored in cyroprotectant (30% (w/v) sucrose, 1% (w/v) Polyvinyl-pyrrolidone, 30% (v/v) ethylene glycol in PBS) to prevent freezing at -20°C until all samples could be processed together in order to allow a robust comparison.

3.2.2 qPCR

RNA was extracted, DNase I treated and converted to cDNA as described in Chapter 2.2. qPCR was carried out on a Corbett rotor gene 6000 (now maintained and produced by Qiagen) using the mastermix template outlined in Chapter 2.2.4. Genes of interest were targeted; these were *Cdkn1c, Nurr1*, *Drd1, Drd2*, *Th, Dat* and *c-fos.* Each reaction was carried out in triplicate for a given sample. The number of samples in each group is indicated in appendix A. The ΔCt was obtained by normalising to the geometric mean Ct value of *βactin* and *Hprt* for that sample. Statistical analysis was carried out on these values. Fold change from wt was calculated using the formula $2^{-\Delta\Delta Ct}$.

3.2.3 Immunohistochemistry and image analysis

Immunohistochemistry was carried out as described in chapter 2.4. Each antibody was applied to 1 in every 5 slices such that the antibodies used, anti-Th and anti-NeuN, were applied to adjacent slices.

3.2.4 High-performance liquid chromatography (HPLC)

Tissue was dissected as described in chapter 2.1.5 and snap frozen and stored at -80°C until further analysis. Tissue was homogenized in 200 µl of 0.2M perchloric acid by an ultrasonic cell disruptor (Microson, UK). Levels of NA, DA, DOPAC, 5-HT and 5-HIAA were determined in the supernatant by reversed-phase, high-performance liquid chromatography, as described previously (Dalley et al., 2001). This was performed by Dr. Jing Xia at the Department Of Psychology, University of Cambridge.

3.2.5 Statistical analysis

For qPCR data Shapiro-Wilk test for normality was carried out on all ΔCt values. Values that were >3 SD from the group were excluded from analysis as outliers. This total was 3 outliers, inclusions of which did not alter any results. Where data was normally distributed independent sample t-tests were carried out. Where the data was not normally distributed non parametric Mann-Whitney U-tests were carried out. To correct for multiple comparisons a Bonferroni corrected α was used for each brain region, namely $\alpha/4=0.0125$. For HPLC repeated measures ANOVA were carried out for each brain region with MOLECULE as within subject variable and GENOTYPE as between-subjects variable. Bonferroni corrections for multiple comparisons were applied and adjusted F and p vales are reported.

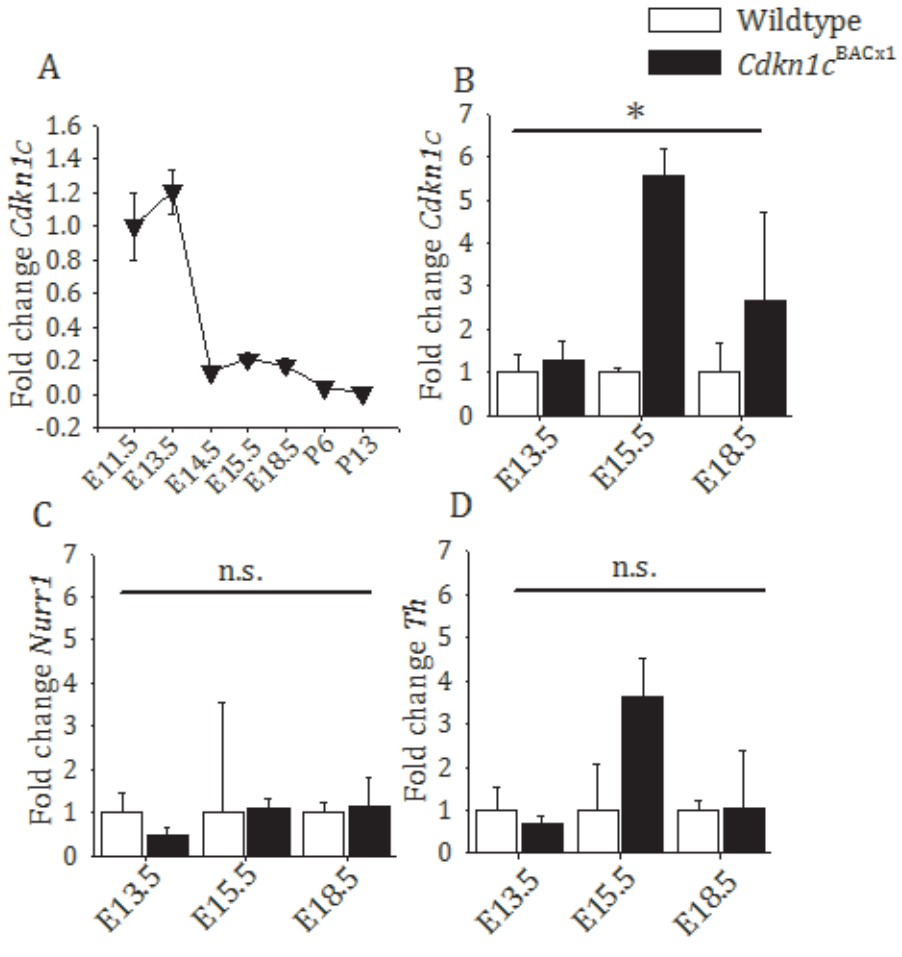
3.3 Results

3.3.1 Embryonic expression profile of Cdkn1c, Nurr1 and Th

qPCR analysis of whole brain revealed a peak in neural *Cdkn1c* expression levels at approximately E13.5 in the wt mouse nervous system and expression declined thereafter (Figure 3.1A). There was a significant increase in *Cdkn1c* expression across E13.5, E15.5 and E18.5 (main effect of GENOTYPE:

$F_{1,3}$=12.364, p=0.039) between *Cdkn1c*^{BACx1} animals and their wt littermates (Figure 3.1B). There was no effect of genotype between *Cdkn1c*BACx1 animals and their wt littermates in expression levels of *Nurr1* (main effect of GENOTYPE: $F_{1,4}$=1.185, p=0.338) (Figure 3.1C) or *Th* (main effect of GENOTYPE: $F_{1,4}$=0.179, p=0.694) (Figure 3.1D).

Figure 3.1: (A) *Cdkn1c* expression peaks at E13.5 in the wt mouse development. *Cdkn1c* expression (B), but not *Nurr1* (C) or *Th* (D), was elevated across embryonic neural development in *Cdkn1c*^{BACx1} embryos relative to wt littermates for each timepoint. n≥4/timepoint. Data shown is mean fold change ± SEM.*p<0.05.



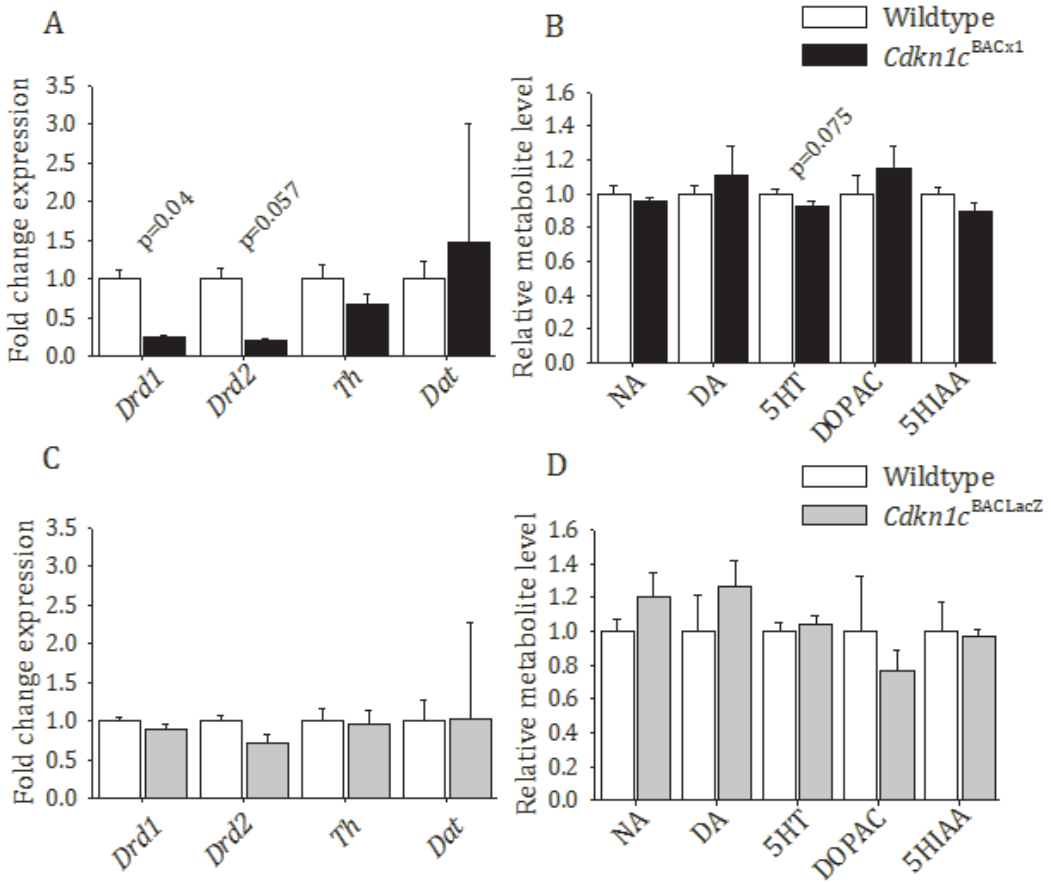
3.3.2 Adult neurochemistry

3.3.2.1 Frontal cortex

Over expression of *Cdkn1c* resulted in a significant decrease in adult frontal cortex expression of *Drd1* (U = 6, p = 0.04) and *Drd2* receptor transcript levels (t(11)=-2.423, p=0.034)(Figure 3.2A). However neither of these values were significant at the Bonferroni adjusted α=0.0125. There was no difference in receptor expression between *Cdkn1c*^{BACLacZ} and their wt littermates (*Drd1*: U=15, p=0.685; *Drd2*: U=14, p =0.57) (Figure 3.2C). There were no significant changes in transcript levels of *Dat* (*Cdkn1c*^{BACx1}: t(10)=-1.075, p=0.308); *Cdkn1c*^{BACLacZ}: t(6)=0.062, p=0.952) or *Th* (*Cdkn1c*^{BACx1} t(11)=-0.685, p=0.507; *Cdkn1c*^{BACLacZ}: t(11)=-0.685, p=0.507) between transgenic animals of either line and their respective wt littermates (Figure 3.2A, C).

Whole tissue neurochemistry analysis revealed no significant differences in any neurotransmitters analysed between *Cdkn1c*^{BACx1} and wt littermates (Figure 3.2B) or between *Cdkn1c*^{BACLacZ} and their wt littermates. (Figure 3.2D). There was a trend for an decrease in 5-HT in *Cdkn1c*^{BACx1} animals ($F_{1,14}$=3.696, p=0.075) (Figure 3.2B) but this did not reach significance (Figure 3.2D). Further work is required to determine the relevance if this observation. Full details of statistical results are available in Appendix A.

Figure 3.2: qPCR (A, C) and HPLC (B, D) analysis of the frontal cortex of *Cdkn1c*^{BACx1} (A, B) and *Cdkn1c*^{BACLacZ} (C, D) adult males. Data shown is mean fold change ± SEM. NA, noradrenaline; DA, dopamine; 5HT, serotonin.

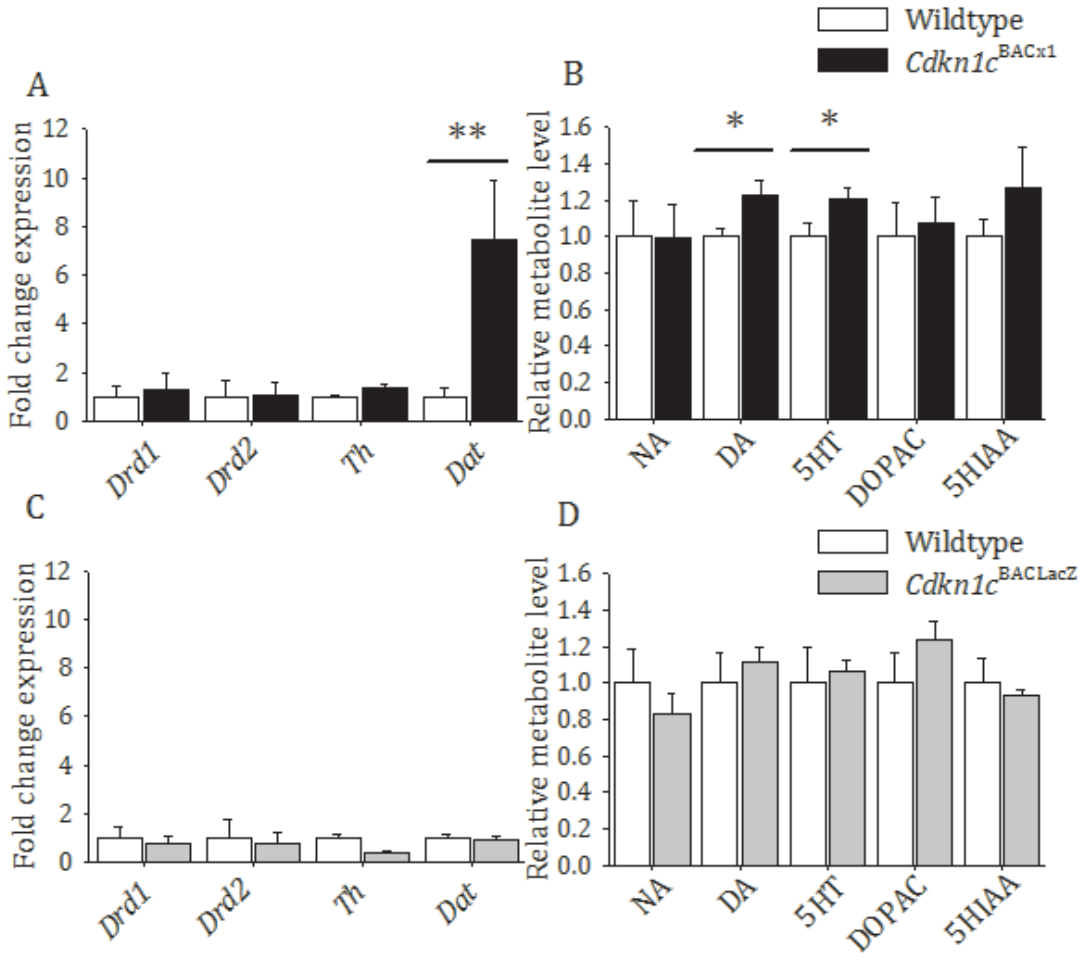


3.3.2.2 Dorsal striatum

Within the dorsal striatum there was no significant change in levels of *Drd1* (t(6)=0.54, p=0.609), *Drd2* (t(6)=0.04, p=0.97) or *Th* (U=5, p=0.386) between *Cdkn1c*^{BACx1} animals and their wt littermates (Figure 3.3A). There was a significant increase in *Dat* (t(5)=4.504, p=0.006) (Figure 3.3A) in this region in animals over expressing *Cdkn1c* at the Bonferroni adjusted α=0.0125. This was consistent with a significant increase in tissue levels of dopamine in the dorsal striatum of *Cdkn1c*^{BACx1} animals ($F_{1,14}$=5.279, p=0.038) (Figure 3.3B). Additionally, there was a significant increase in serotonin in this region ($F_{1,14}$=5.102, p=0.04) (Figure 3.3B). There was no significant difference in *Drd1* (t(7)=-0.916, p=0.39), *Drd2* (t(7)=-0.915, p=0.391), *Th* (t(6)=-2.247, p=0.066) or *Dat* (t(8)=-0.1, p=923) expression (Figure 3.3C) nor was there a difference in

tissue levels of dopamine ($F_{1,14}$=0.481, p=0.499) or serotonin ($F_{1,14}$=0.178, p=0.679) in this region between *Cdkn1c*^{BACLacZ} and their wt littermates (Figure 3.3D).

Figure 3.3: qPCR (A, C) and HPLC (B, D) analysis of the dorsal striatum of *Cdkn1c*^{BACx1} (A, B) and *Cdkn1c*^{BACLacZ} (C, D) adult males. Data shown is mean fold change ± SEM. *p<0.05 **p<0.01. NA, noradrenaline; DA, dopamine; 5HT, serotonin.

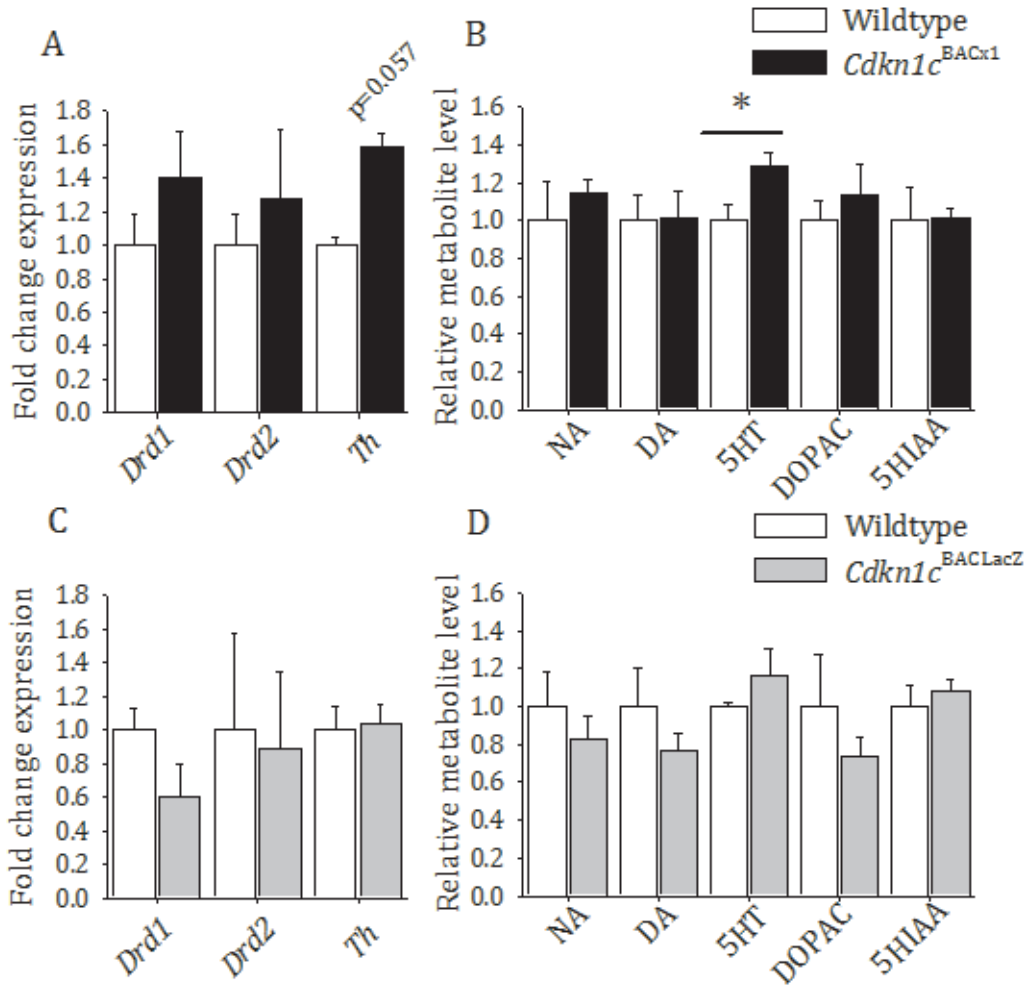


3.3.2.2 Ventral striatum

In the ventral striatum there was no significant differences between animals over expressing *Cdkn1c* and their wt littermates in the expression of *Drd1* (t(9)=1.097, p=0.301), *Drd2* (t(9)=0.615, p=0.554) or *Dat* (t(4)=-0.343, p=0.728) (Figure 3.4A). There was a trend for an increase of *Th* expression in this region

(t(9)=2.013, p=0.075) (Figure 3.4A). There was no difference in whole tissue levels of dopamine between *Cdkn1c*^{BACx1} animals and their wt littermates in this region ($F_{1,14}$=0.308, p=0.588) (Figure 3.4B). There was a significant increase in 5-HT in *Cdkn1c*^{BACx1} animals, after adjustment for multiple comparisons ($F_{1,14}$=6.686, p=0.022) (Figure 3.4B). This was not the case between *Cdkn1c*^{BACLacZ} and their wt littermates ($F_{1,14}$=1.471, p=0.245) (Figure 3.4D). There was no significant change in *Drd1* (t(5)=-0.920, p=0.4), *Drd2* (t(5)=-0.22, p=0.834) or *Th* (t(5)=-0.088, p=0.933) between *Cdkn1c*^{BACLacZ} and their wt littermates (Figure 3.4C). Additionally, there was no difference in tissue levels of dopamine in this region between both *Cdkn1c*^{BACx1} and their wt littermates ($F_{1,14}$=0.007, p=0.934) and *Cdkn1c*^{BACLacZ} and their wt littermates ($F_{1,14}$=0.349, p=0.564) (Figure 3.4D).

Figure 3.4: qPCR (A, C) and HPLC (B, D) analysis of the ventral striatum of *Cdkn1c*^{BACx1} (A, B) and *Cdkn1c*^{BACLacZ} (C, D) adult males. Data shown is mean fold change ± SEM. *p<0.05. NA, noradrenaline; DA, dopamine; 5HT, serotonin.

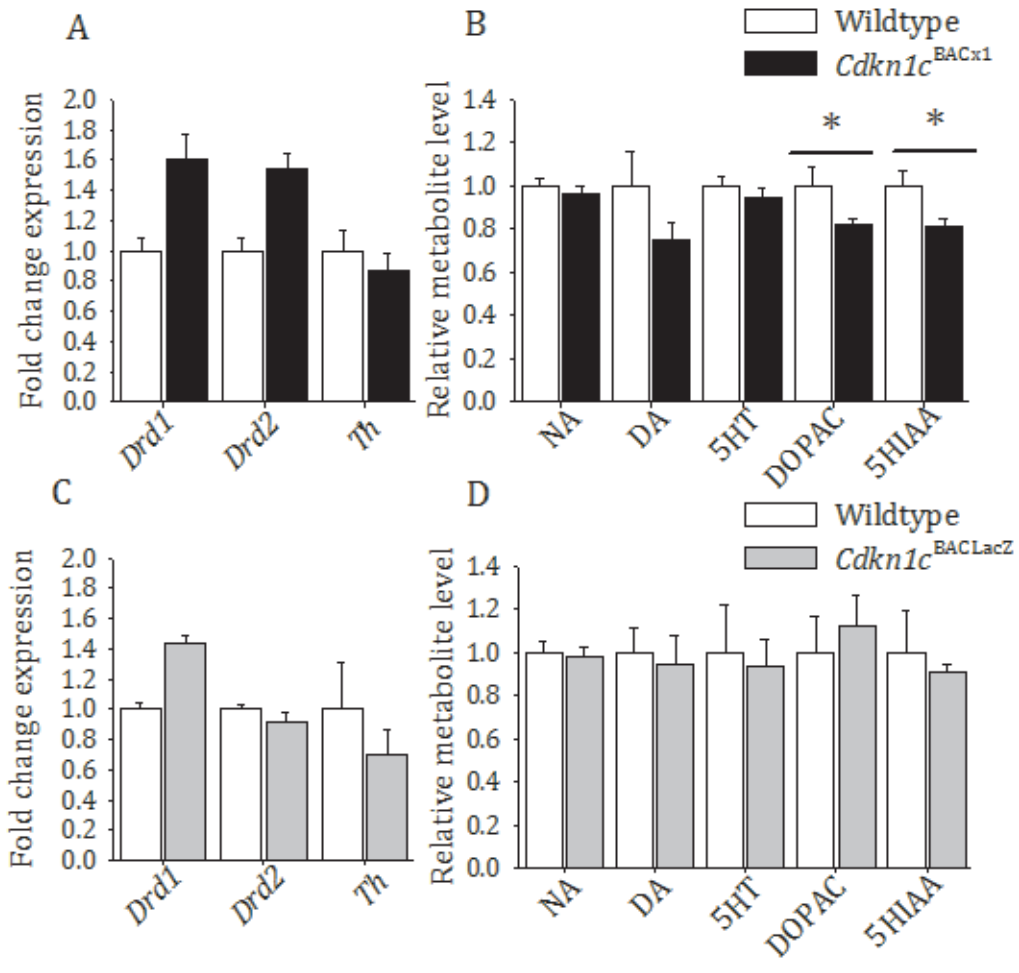


3.3.2.4 Hypothalamus

There was no effect of genotype of either line on expression levels of *Drd1* (*Cdkn1c*^{BACx1}: t(5)=1.102, p=0.321; *Cdkn1c*^{BACLacZ}: t(5)=1.959, p=0.107), *Drd2*(*Cdkn1c*^{BACx1}: t(5)=1.695, p=0.151; *Cdkn1c*^{BACLacZ}: t(5)=-0.374, p=0.724) or *Th*(*Cdkn1c*^{BACx1}: t(6)=-0.426, p=0.685; *Cdkn1c*^{BACLacZ}: t(6)=-0.447, p=0.671) in the hypothalamus (Figure 3.5A, C). There was no difference in the whole tissue level of dopamine ($F_{1,14}$=2.222, p=0.158) or 5-HT in this region ($F_{1,14}$=0.211, p=0.409) between *Cdkn1c*^{BACx1} animals and their wt littermates (Figure 3.5B). However, there was a significant decrease in DOPAC and 5HIAA, metabolites of both of these neurotransmitters, respectively (DOPAC: $F_{1,14}$=4.99, p=0.042;

5HIAA $F_{1,14}$=6.948, p=0.02) (Figure 3.5B), implying reduced turnover in this region. None of these measurements differed between *Cdkn1c*^{BACLacZ} animals and their wt littermates (dopamine: $F_{1,14}$=0.05, p=0.827; 5-HT: $F_{1,14}$=0.075, p=0.788; DOPAC: $F_{1,14}$=0.23, p=0.639; 5HIAA: $F_{1,14}$=0.566, p=0.464) (Figure 3.5C, D).

Figure 3.5: qPCR (A, C) and HPLC (B, D) analysis of the hypothalmus of *Cdkn1c*^{BACx1} (A, B) and *Cdkn1c*^{BACLacZ} (C, D) adult males. Data shown is mean fold change ± SEM. *p<0.05. NA, noradrenaline; DA, dopamine; 5HT, serotonin.

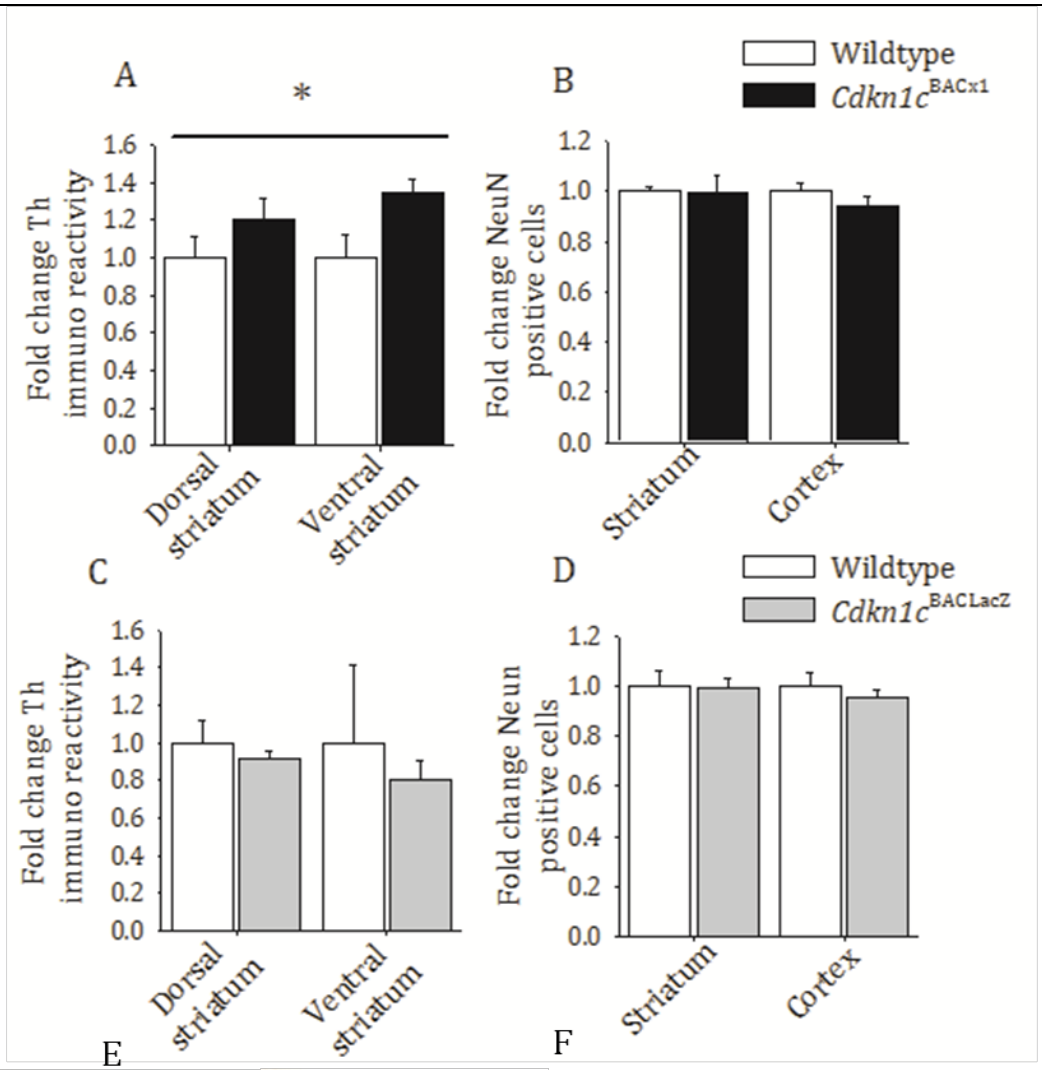


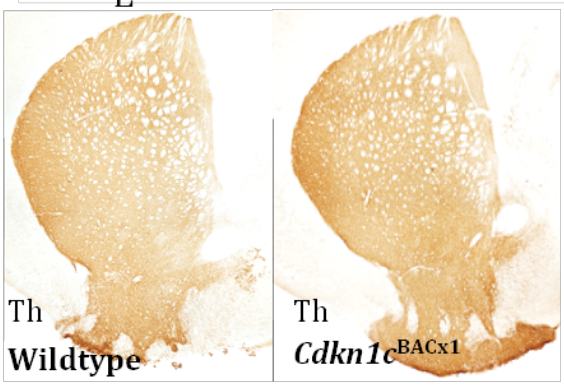
3.3.3 Adult neuroanatomy

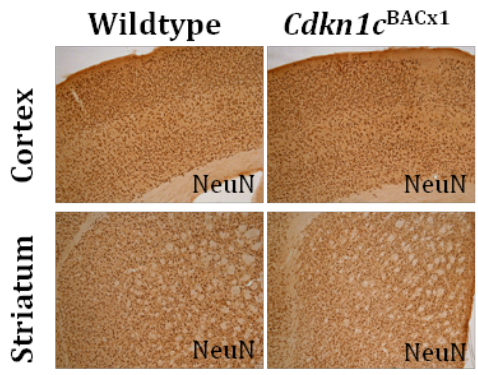
There was no effect of genotype on the average number of neurons in the striatum or surrounding cortex between *Cdkn1c*^{BACx1} and their wt littermates (striatum: $F_{1,9}$=0.001, p=0.982 ; cortex: $F_{1,9}$=1.33, p=0.282) (Figure 3.6B) or between *Cdkn1c*^{BACLacZ} and their wt littermates (striatum: $F_{1,7}$=0.025, p=0.879 ;

cortex: $F_{1,7}$=0.732, p=0.425) (Figure 3.6D). However, there was a significant increase in Th staining intensity in the dorsal and ventral striatum in *Cdkn1c*^{BACx1} animals compared to their wt littermates (Main effect of GENOTYPE: $F_{1,6}$=7.958,p=0.03) (Figure 3.6A) but not between *Cdkn1c*^{BACLacZ} animals compared to their wt littermates (Main effect of GENOTYPE: $F_{1,6}$=0.617,p=0.462) (Figure 3.6C).

Figure 3.6: Th immuno-reactivity (A, C) and NeuN^{+} cell count (B, D) analysis of the striatum and cortex of *Cdkn1c*^{BACx1} (A, B) and *Cdkn1c*^{BACLacZ} (C, D) adult males. E) Representative images showing Th immuno-reactivity in striatum of *Cdkn1c*^{BACx1} and wt littermate. F) Representative images showing NeuN staining in striatum and adjacent cortex of *Cdkn1c*^{BACx1} and wt littermate. Data shown is mean fold change ± SEM. *p<0.05.



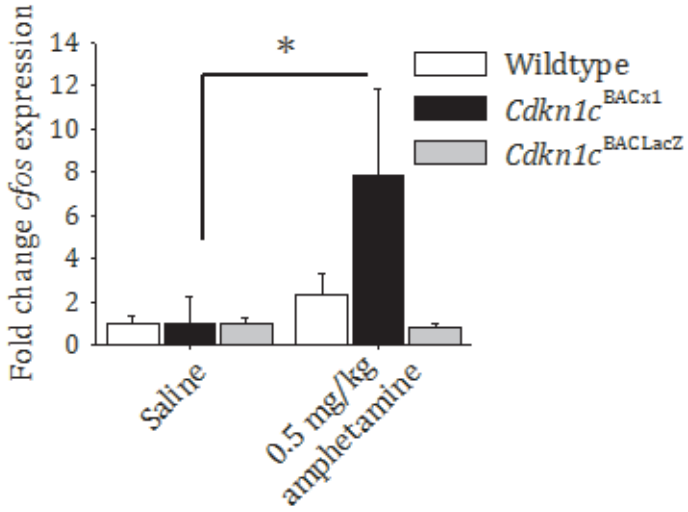




3.3.4 Cdkn1c^{BACx1} amphetamine hypersensitivity

60 minutes after a single i.p. injection of amphetamine there was a significant increase in *cfos* expression in the nucleus accumbens of all animals over expressing *Cdkn1c* (*Cdkn1c*^{BACx1}: $F_{1,9}$=6.214, p=0.037) (Figure 3.7. The increase was not significant in their wt littermates ($F_{1,11}$=3.162, p=0.106)), nor in *Cdkn1c*^{BACLacZ} animals ($F_{1,9}$=0.364, p=0.563) (Figure 3.7). This implies that *Cdkn1c*^{BACx1} animals were more sensitive to amphetamine in the nucleus accumbens than animals not over expressing *Cdkn1c*.

Figure 3.7: *Cdkn1c*^{BACx1} animals had increased neural activity in response in the nucleus accumbens in response to a low dose of i.p. amphetamine compared to wt littermates and *Cdkn1c*^{BACLAcZ} animals. Data shown is mean fold change ± SEM. *p<0.05.



3.4 Discussion

This chapter focussed on the neurochemical characterisation of animals over expressing *Cdkn1c*. Neural over-expression of *Cdkn1c* in *Cdkn1c*^{BACx1} animals was demonstrated at several timepoints, as has been shown previously at E12.5 (Andrews et al., 2007). Regional changes in gene expression and neurochemistry were identified in the adult brain between *Cdkn1c*^{BACx1} animals and their wt littermates but not between *Cdkn1c*^{BACLacZ} animals and their wt

littermates attributing these changes to elevated *Cdkn1c*. Finally, animals over expressing *Cdkn1c* were found to be hypersensitive to a low dose of amphetamine in the nucleus accumbens, as indicated by a significant increase in *cfos* expression after injection compared to the wt littermates. These results demonstrated that *Cdkn1c* gene dosage contributes to neural development and impacts adult neurochemistry and neural activity.

3.4.1 Embryonic Cdkn1c and dopaminergic system development

A rapid decline in *Cdkn1c* expression from E13.5 to P13 occurred in the developing wt brain. As has previously been shown at E12.5 (Andrews et al., 2007), *Cdkn1c* expression was higher in the whole brains of *Cdkn1c*^{BACx1} embryos compared to their wt littermates. *Nurr1* expression was not significantly different in response to elevated *Cdkn1c* at any time point examined. *Nurr1* is expressed in the developing and adult nervous system and co-localises with *Th*, though expression during development proceeds *Th* (Zetterström et al., 1996). *Cdkn1c* gene is a target of *Nurr1* and *Cdkn1c* expression in the developing ventral midbrain is dependent on presence of *Nurr1* (Joseph et al., 2003). Together these two genes cooperate to promote the proliferation of midbrain dopaminergic neurons (Joseph et al., 2003). Absence of *Nurr1* results in a loss of striatal dopamine (Zetterström et al., 1997). In contrast, *Cdkn1c* null embryos retain Th immuno-reactive cells at E18.5 (Joseph et al., 2003). Given that *Nurr1* acts upstream of *Cdkn1c*, at least in this system, it is not expected that *Nurr1* would be altered following *Cdkn1c* over expression. Genetic ablation of *Cdkn1c* causes a regional specific decrease in *Nurr1* (Joseph et al., 2003), which given the relatively crude dissection in this study (whole brain), may not be possible to discriminate here. Similarly for *Th* expression, the dissection method may be too crude to discern any region specific changes, especially given the relatively restricted expression of the transgene in the nervous system (John et al., 2001). Additionally, *Th* is involved in the synthesis of not just dopamine but also noradrenaline, a neurotransmitter not altered in the adult brains of *Cdkn1c*^{BACx1} animals and this may contribute to an increased variability when examining whole brain samples.

3.4.2 Adult neurochemistry of Cdkn1c^{BACx1} and Cdkn1c^{BACLacZ} lines

3.4.2.1 Frontal cortex

Dopamine in the frontal cortex is multifunctional (Beaulieu and Gainetdinov, 2011) and the relatively crude whole tissue dissection used here means it is not possible to discern which activities the decrease in Drd1 and Drd2 expression may affect. Chronic treatment of rats with a Drd1 antagonist leads to up regulation of levels of this receptor in the striatum (Hess et al., 1986) implying that decreased activation of this receptor may lead to an up regulation. Conversely, and relevant for this work, it is possible that increased dopamine receptor activation, as a result of a hyper-dopaminergic mesocortical input, may cause a reactive down regulation in these receptors. There was no observed difference in whole tissue levels of dopamine or in expression of the *Th* or *Dat,* in this study but it is not possible to rule out whether enhanced dopamine release, either basally or upon stimulation, causes the observed down regulation in receptor levels in *Cdkn1c*^{BACx1} animals.

3.4.2.2 Dorsal and ventral striatum

The observation that animals over expressing *Cdkn1c* have higher levels of dopamine than their wt littermates in the dorsal striatum is consistent with the role of *Cdkn1c* in promoting the proliferation of midbrain dopaminergic neurons (Joseph et al., 2003; Freed et al., 2008). A higher level of dopamine was accompanied by an increase in Th immuno-reactivity in the striatum and a decrease in *Dat* levels in the dorsal striatum. Together, this would be expected to increase tissue levels of dopamine by increasing synthesis and decreasing reuptake for degradation. A significant increase in *Th* expression was not observed at the mRNA level in *Cdkn1c*^{BACx1} animals compared to their wt littermates. The increase in protein immuno-reactivity however implies that there is functional consequence to the increased expression of *Cdkn1c* in *Cdkn1c*^{BACx1} adult males. Importantly, given the role of *Cdkn1c* in the cell cycle, there were no differences between *Cdkn1c*^{BACx1} animals and their wt littermates in neuron number in the striatum and surrounding cortex. Interestingly, there

was a significant increase in serotonin in both the dorsal and ventral striatum. *Cdkn1c* is not known to have direct activity on the serotoninergic system development. However, dopamine has been shown to promote serotonin release in the hippocampus in a Drd2 dependant manner (Matsumoto et al., 1996). Additionally L-DOPA (the dopamine precursor) perfusion into the substantia nigra caused an increase in extra neuronal serotonin levels in the same region and also in the striatum (Thorre et al., 1998). In addition to dopamine facilitated serotonin release, the converse has also been shown to occur. In the striatum serotonin perfusion (Benloucif and Galloway, 1991) and in the prefrontal cortex serotonin increase mediated through a reuptake inhibitor (Matsumoto et al., 1999), both increased extracellular dopamine. Taken together, this implies that the *Cdkn1c* mediated increase in dopamine may cause changes in the serotonin system, which in turn may increase dopamine-potentiating phenotypes.

A low dose of amphetamine was used to probe the sensitivity of animals over expressing *Cdkn1c* to this compound, as an enhanced sensitivity may be expected from other studies on hyper-dopaminergic animals (O'Neill and Gu, 2013). Much higher doses than presented here are classically used to induce *cfos* expression in the ventral striatum (Graybiel et al., 1990). The observed significant *cfos* induction after amphetamine injection in animals over expressing *Cdkn1c* implies these animals are neurally hypersensitive to rewarding stimulants. The location of activation, the ventral striatum, is of particular significance. This contains the nucleus accumbens, a primary constituent in the mesolimbic reward pathway. This implies that *Cdkn1c*$^{\mathrm{BACx1}}$ may have altered processing of rewarding stimuli.

3.4.2.3 Hypothalamus

There was no change in the hypothalamus in expression of any of the genes examined in either line. However, there was a significant decrease in the metabolites of dopamine and serotonin degradation but without a change in levels of the neurotransmitters themselves. This was specific to animals over expressing *Cdkn1c*. This finding has several possible interpretations. There may

be reduced release of these neurotransmitters, something that is not possible to gauge from crude tissue levels as these values include transmitters stored in vesicles. Another possibility is that there is a reduced rate of synthesis and/or reuptake of these neurotransmitters. While *Th* and *Dat* as well as *Tph2* and *5htt* (not shown here) mRNA levels were normal, this does not exclude the possibility of reduced protein levels. Alternatively, there may be a reduction in the levels of the monoamine oxidase A, the common degrading enzyme for both dopamine and serotonin, in the hypothalamus (Jahng et al., 1997). Further work is required to investigate this cause of the altered metabolism.

3.4.3 Conclusions

Transgenic over expression of *Cdkn1c* mediated through BAC insertion produces a relatively moderate, embryonically sustained, increase in expression of this gene in the brain. Importantly, this increased expression produced robust phenotypes in both the neurochemistry and neural responsively of the adult brain. The consequences for adult neurochemistry occurred in the region dependant manner. Additionally, neurotransmitter systems not shown to be directly influenced by increased *Cdkn1c* expression *per se*, namely the serotoninergic system, also showed alterations in the adult *Cdkn1c*$^{\mathrm{BACx1}}$ male brain.

In conclusion, this work revealed that animals over expressing Cdkn1c were hyper-dopaminergic both in terms of their basal neurochemical state and in their response to external stimuli. This study highlighted the importance of correct *Cdkn1c* dosage during development for adult neural functioning and suggested a behavioural phenotypic consequences of this alteration in *Cdkn1c* expression.

Chapter 4: Basic behavioural characterisation

4.1 Introduction

This chapter concerns the initial behavioural characterisation of male mice overexpressing *Cdkn1c* (*Cdkn1c*^{BACx1}) alongside the control line (*Cdkn1c*^{BACLacZ}). The assessment of basic motoric and anxiolytic function of these animals was made to exclude the possibility that more basic aspects of behaviour were compromised and to eliminate these as a potential confounders during further testing (Crawley, 1999; Sousa et al., 2006).

The dopaminergic cells of the midbrain have a crucial role in regulating movement (Groenewegen, 2003). For instance, drugs that increase midbrain dopamine concentrations, such as cocaine (Gainetdinov, 1999; Walker et al., 2001; Wyvell and Berridge, 2001; Zhuang et al., 2001; Napolitano et al., 2010; Vucetic et al., 2010b; Yu et al., 2014), amphetamine (Gainetdinov, 1999; Napolitano et al., 2010; Flores et al., 2011; O'Neill and Gu, 2013) or methylphenidate (Gainetdinov, 1999; Carmack et al., 2014) have been shown to cause hyperactivity in rodents. Additionally, genetically hyper-dopaminergic animals resulting from knock down of the *Dat*, were found to have altered activity levels (Gainetdinov, 1999; Zhuang et al., 2001; Napolitano et al., 2010; O'Neill and Gu, 2013). As shown in chapter 3, animals over-expressing *Cdkn1c* were hyper-dopaminergic in the dorsal striatum, with dysregulation of a number of genes in the dopaminergic system in the striatum, as well as in the frontal cortex. Given the potential confound of any affect on locomotor activity on other aspects of behaviour, it was necessary to assess motor learning and general activity levels of animals over-expressing *Cdkn1c*.

Similarly, anxiety related behaviours have been shown to be sensitive to manipulations of the dopaminergic system. A number of *DAT* variants were recently found to be associated with emotional dysregulation in children, specifically in those with generalized anxiety (Gadow et al., 2014). Additionally, at therapeutic doses the anxiolytic drug, busiprone, has been shown to bind to DRD3 and DRD4 receptors with an affinity similar to its classic target, the

5HT1a receptor (Bergman et al., 2013; Kim et al., 2014). *Cdkn1c* over expression decreased in *Drd1* expression in the frontal cortex (Figure 3.2A). Virally mediated over expression of DRD1 in glutamatergic neurons of the prefrontal cortex in adult rodents resulted in decreased anxiety levels (Sonntag et al., 2014). Importantly, another maternally expressed imprinted gene, *Nesp55*, has previously been shown to influence aspects of this behaviour (Plagge et al., 2005). Given that differences in novelty reactivity and/or anxiety could influence behaviours in a variety of behavioural tasks, it was necessary to assess any baseline difference as a result of altering the dosage of *Cdkn1c*.

As a 'proof of principle' of altered dopaminergic function it is possible perform experiments known to be sensitive to the levels of dopamine of an individual. Locomotor activity in response to a single injection of a low dose of amphetamine is one such task. Amphetamine acts as a competitive inhibitor of dopamine (and other monoamines), both at the dopamine transporter and at the vesicular monoamine transporter, via its inhibitory effect on TAAR1 (Miller, 2011). This causes an increase of extracellular dopamine. Genetically hyper-dopaminergic animals have been previously shown to be hypersensitive to this drug at doses at which wt animals were not affected, as indicated by differences in locomotor activity (O'Neill and Gu, 2013).

Similarly pre-pulse inhibition (PPI) of the startle response is sensitive to neuronal levels of dopamine. Both rodents and humans have an involuntary motoric response to an unexpected 'startling' acoustic noise, this is known as the acoustic startle response (ASR). This response is blunted when the pulse is preceded by a pre-pulse that does not evoke a response by itself (Groves et al., 1974; Graham, 1975; Frost et al., 2003). A reduced ability to inhibit responding to a startling acoustic noise, when preceded by a quieter pre-pulse, is characteristic of a hyper-dopaminergic state, both in humans (Hutchison and Swift, 1999) and animals (Ralph et al., 2001). The dopamine agonist apomorphine decreases the percentage inhibition in rodents (Kanno et al., 2014) as well as antagonists of dopamine D_1 and D_2 receptors (Schwarzkopf et al., 1993; Swerdlow et al., 2004; Swerdlow et al., 2005) and both typical and

atypical antipsychotics restore PPI deficits in apomorphine treated rodents (Swerdlow et al., 1994).

This chapter concerns the basic behavioural, motoric, and anxiolytic characterisation of *Cdkn1c*^{BACx1} animals and their wt littermates, and *Cdkn1c*^{BACLacZ} animals and their wt littermates. In addition, characteristic dopaminergic state reporter assays were carried out in these two lines. PPI and ASR were assessed in each animal as a measure of dopaminergic system function. Finally sensitivity to amphetamine was assayed in a locomotor activity task after injection of a sub-stimulatory dose of amphetamine.

4.2 Methods

4.2.1 Animals

109 male mice in total were used for open field and elevated plus maze tasks, genotype: *Cdkn1c*^{BACx1} (n=29) and wt littermates (n=27), *Cdkn1c*^{BACLacZ} (n=31) and wt littermates (n=22). A subset of these was tested in the remaining experiments, genotype: *Cdkn1c*^{BACx1} (n=15) and wt littermates (n=16), *Cdkn1c*^{BACLacZ} (n=16) and wt littermates (n=14). All animals were between 7 and 9 weeks at the start of testing. General housing and husbandry conditions were as described in Chapter 2.1. Food and water were provided *ad lib* for the duration of testing.

4.2.2 Anxiety related behaviours

4.2.2.1 Open field (OF)*:*

The OF apparatus (750 x 750 mm) was constructed of opaque perspex and illuminated evenly with a 60w bulb. The OF arena was divided into 2 virtual zones, an inner (central 450 x 450 mm) and an outer (150 mm periphery). Animals were allowed to explore the arena freely for 600 s. Activity was tracked using a camera connected to a computer with ETHOVISION software (Noldus, UK) and time spent in and entries made into each zone, as well as overall distance moved, was recorded. Greater time spent in the inner zone was considered less anxious behaviour.

4.2.2.2 Elevated plus maze

The elevated plus maze (EPM) consisted of four Perspex arms two open (175 x 78 mm) and two enclosed (190 x 80 x 150 mm) with an open roof. The EPM was 940 mm above the floor and illuminated evenly by a 60 W bulb. Animals were placed centrally at the beginning of a trial and allowed to explore freely for 300 s. Activity was tracked using a camera connected to a computer with ETHOVISION software (Noldus, UK) and time spent in and entries made into each arm was recorded. The main measures of anxiety related behaviour were time spent on, and number of entries into, the open arm.

4.2.3 Motoric function

4.2.3.1 Rotarod

A rotarod task (Ugo Basile, Italy) was used to assess motor learning and co-ordination. This consisted of a rotating rod 30 mm in diameter, with five separated chambers 57 mm in width, with a rod elevation of 160 mm. Motor learning was assessed across five sessions in which rod speed accelerated linearly from 5 to 50 rpm across 300 s. Latency to first fall was recorded for each session. After each fall, the animal was replaced onto the rod until the end of the session. The main measure of motor co-ordination was latency to first fall.

4.2.3.2 Locomotor activity

Locomotor activity (LMA) behaviour was tested using an apparatus consisting of twelve Perspex chambers (210 x 360 x 200 mm), with 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 indication of activity, using a computer running custom written BBC Basic V6 programmes with additional interfacing by ARACHNID (Cambridge Cognition Ltd, U.K.). Data stored were the total number of beam-breaks in a 2 hour session, as well as the number of beam-breaks made in each 5 minute bin. One session was carried out per day, for three consecutive days as a measure of habituation to a novel environment, both within, and across, sessions. The main measure of activity was number of beam breaks made across the whole session, as well as in each five minute bin.

Habituation to a novel arena was assayed by the decrease in beam breaks across sessions or across time bins within a session.

Following the three days of basal LMA, animals were injected intra-peritoneally (i.p.) with saline or a sub-stimulatory dose of D-amphetmine sulphate (Tocris Bioscience, UK), either 0.5 mg/kg or 1 mg/kg to detect any enhanced sensitivity of to amphetamine. All animals received each solution in a pseudo-randomised order, with 72 hours between each injection to allow for solution wash out. Session total beam breaks were used as an indication of amphetamine induced LMA.

4.2.4 Acoustic Startle (ASR) and Prepulse Inhibition (PPI)

ASR and PPI were monitored using a SR-Lab apparatus (San Diego Instruments, U.S.A) modified for use in mice. White noise stimuli were presented via a speaker mounted in the roof of a sound-attenuating chamber, 120mm above the subject. Animals were placed in a Perspex tube (internal diameter 35mm) mounted onto a Perspex plinth. A session consisted of a 5min habituation period followed by 3 blocks of acoustic stimuli presented against a background white noise set to 70db (A scale) with the startle amplitude set to 120db in the first block, 105db in the second and a range (80 to 120db) in the 3rd. Pulse-alone trials consisted of a 40ms startle stimulus and a prepulse trial consisted of a 20ms prepulse at 4, 8, or 16db above background and a 40ms, 120db or 105db startle stimulus, 70ms after the prepulse offset. In blocks 1 and 2, following 5 pulse-alone trials, there followed five blocks consisting of 2 pulse-alone trials, 1 no stimulus trial and 6 prepulse trials (2 each of 4, 8 and 16db above background). In each block, the different stimuli were presented in a pseudorandom manner every 15s. The whole body startle response to the pulse alone trials and the gating (i.e. inhibition) of responding due to the presentation of prepulse stimuli was recorded as the average startle during a 65ms window timed from the onset of the startle pulse. Values were transduced and digitised by a piezoelectric transducer linked to the computer. PPI was calculated as the percentage reduction in startle between prepulse trials and pulse alone trials.

Response output was adjusted for weight as described previously (Doe et al., 2009; Relkovic et al., 2010).

4.2.5 Statistical analysis

All statistical analysis was carried out using SPSS 20 (SPSS, USA). All analysis was carried out separately for each line, followed by comparison between the wt animals of each line. Sphericity of the data was analysed using Mauchly's test and where this was violated degrees of freedom was adjusted using Greenhouse-Geisser adjustment.

For EPM and OF tasks a series of one-way ANOVAs were carried out to assess the effect of genotype on duration of trial spent in each zone, factors were TIME SPENT IN OPEN ARM and TIME SPENT IN INTERNAL ZONE, respectively. Preference for the less anxiogenic zone (closed arm and external zone, respectively) was assessed in both tasks using a paired samples t-test. For further OF analysis additional ANOVAs were carried out to exclude any confounding factors such as DISTANCE MOVED. A repeated measure ANOVA was carried out to examine any difference in the rate of habituation to a novel environment, DISTANCE MOVED MIN1-10 were used as within subject factors.
Rotarod performance was analysed using a repeated measures ANOVA with LATENCY TO FIRST FALL as a within-subjects factor across training sessions and GENOTYPE as the between subjects factor.

LMA habituation trials were analysed using a series of repeated measure ANOVAs for each session with SESSION or BIN as the within-subjects factor and GENOTYPE as the between session factor. Amphetamine induced LMA was analysed using a repeated measure ANOVA with DRUG and BIN as the within-subject factor and GENOTYPE as the between subjects factor, this allowed examination of difference in LMA and any difference in rate of change of behaviour.

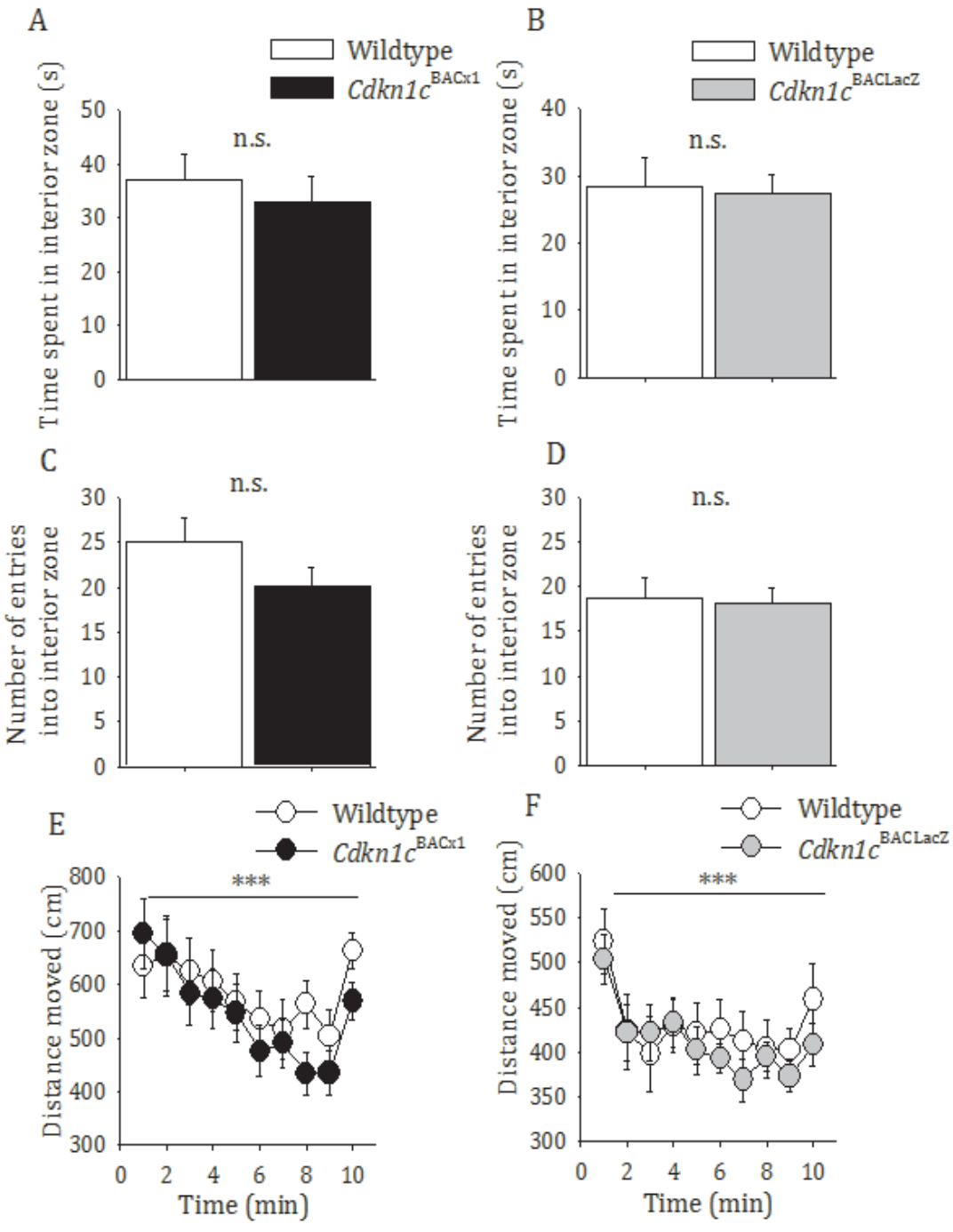
ASR and PPI was analysed using a repeated measures ANOVA with TRIAL and INTENSITY, respectively, as a within-subjects factor and GENOTYPE as the between subjects factor.

4.3 Results

4.3.1 Open field

As expected, all animals preferred the exterior zone to the internal zone, as indicated by increased time spent in this zone (*Cdkn1c*^{BACx1}: t(54)=77.456, p<0.001; *Cdkn1c*^{BACLacZ}: t(55)=111.386, p<0.001). There was no significant effect of genotype on the duration of time spent in the internal zone, between *Cdkn1c*^{BACx1} animals and their wt littermates (Main effect of GENOTYPE: $F_{1,54}$= 0.349, p=0.557) (Figure 4.1A) or between *Cdkn1c*^{BACLacZ} animals and their wt littermates (Main effect of GENOTYPE: $F_{1,55}$= 0.057, p=0.812) (Figure 4.1B). There was no significant effect of genotype on the frequency of entries made into the internal zone, between *Cdkn1c*^{BACx1} animals and their wt littermates (Main effect of GENOTYPE: $F_{1,54}$= 1.996, p=0.16) (Figure 4.1C) or between *Cdkn1c*^{BACLacZ} animals and their wt littermates (Main effect of GENOTYPE: $F_{1,55}$= 0.034, p=0.86) (Figure 4.1D). All animals habituated to the arena across the 600 s trial, as indicated by a decrease in distance moved per minute (Main effect of TIME BIN: *Cdkn1c*^{BACx1}: $F_{9,477}$=11.554, p<0.001; *Cdkn1c*^{BACLacZ}: $F_{9,486}$=5.778, p<0.001), and there was no effect of genotype on either total distance moved (*Cdkn1c*^{BACx1}: $F_{1,54}$=0.402, p=0.529; *Cdkn1c*^{BACLacZ}: $F_{1,54}$=0.338, p=0.563), nor rate of habituation (TIME BIN*GENOTYPE interaction: *Cdkn1c*^{BACx1}: $F_{9,477}$=1.591, p=0.115; *Cdkn1c*^{BACLacZ}: $F_{9,486}$=0.576, p=0.817) (Figure4.1E,F).

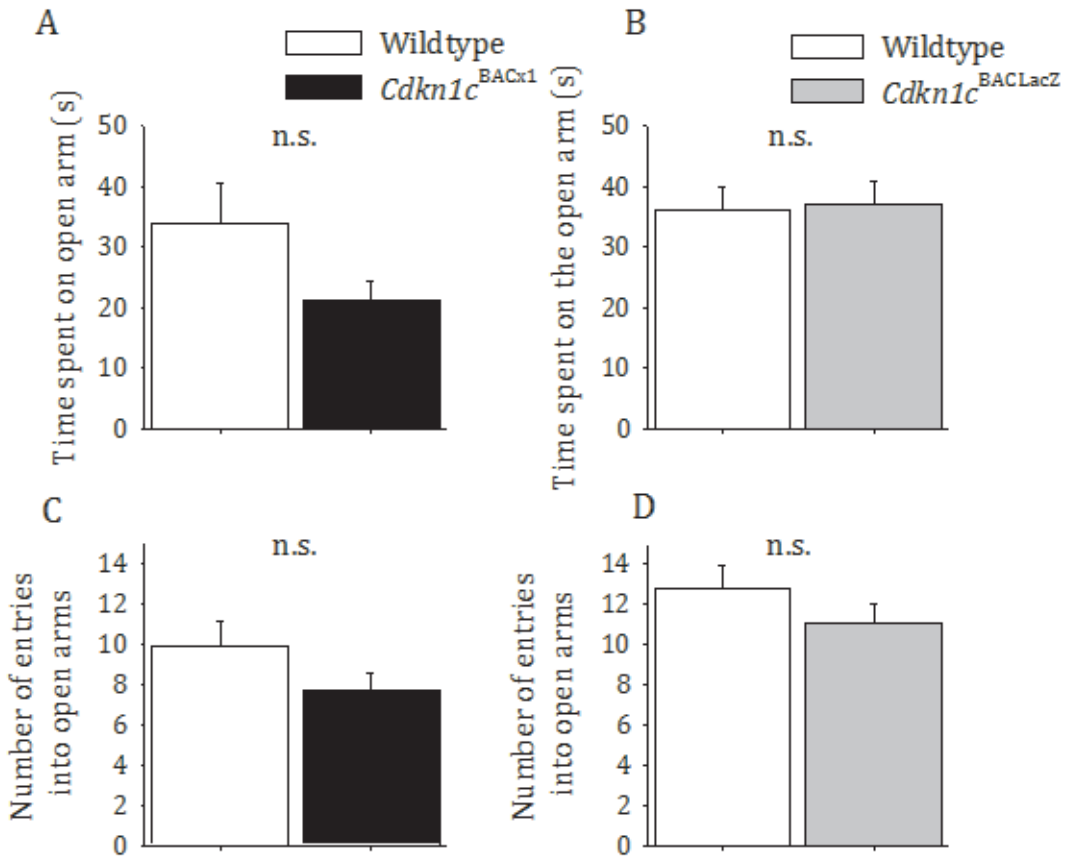
Figure 4.1: There was no effect of genotype on anxiety related behaviours or rate of habituation in an open field task. A, B) There was no difference between *Cdkn1c*^{BACx1} animals and their wt littermates (A) or between *Cdkn1c*^{BACLacZ} animals and their wt littermates (B) in time spent in the anxiogenic interior zone of an open field. C,D) There was no difference in distance travelled per minute between *Cdkn1c*^{BACx1} animals and their wt littermates or between *Cdkn1c*^{BACLacZ} animals and their wt littermates. All data is displayed ± SEM. *** p<0.001



4.3.2 Elevated plus maze

All animals preferred the closed to the open arms, as indicated by increased time spent in these arms, as would be expected (*Cdkn1c*^{BACx1}: t(54)=21.63, p<0.001; *Cdkn1c*^{BACLacZ}: t(55)=20.321, p<0.001). This was not different by genotype as there was no effect of genotype on the duration of time spent in the open arms across the 300 s trial between *Cdkn1c*^{BACx1} animals and their wt littermates (Main effect of GENOTYPE: $F_{1,55}$= 2.99, p=0.09) (Figure 4.2A) or between *Cdkn1c*^{BACLacZ} animals and their wt littermates (Main effect of GENOTYPE: $F_{1,55}$= 0.019, p=0.89) (Figure 4.2B). There was also no effect of genotype on frequency of entries made into the open arms between *Cdkn1c*^{BACx1} animals and their wt littermates (Main effect of GENOTYPE: $F_{1,55}$= 2.212, p=0.14) (Figure 4.2C) or between *Cdkn1c*^{BACLacZ} animals and their wt littermates (Main effect of GENOTYPE: $F_{1,55}$= 1.328, p=0.25) (Figure 4.2D).

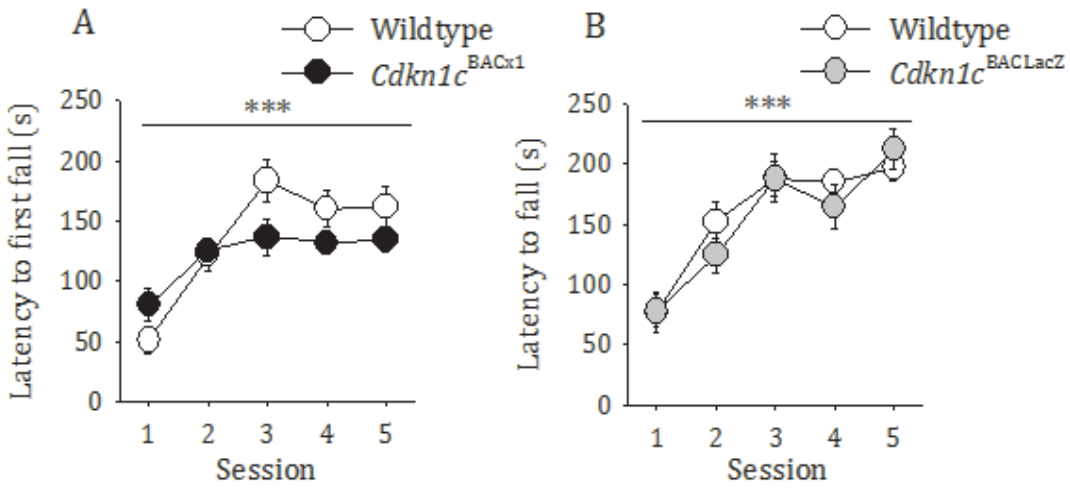
Figure 4.2: There was no effect of genotype on anxiety related behaviours elevated plus maze task. A, B) There was no difference between *Cdkn1c*^{BACx1} animals and their wt littermates (A) or between *Cdkn1c*^{BACLacZ} animals and their wt littermates (B) in time spent in the anxiogenic open arms of an EPM. All data is displayed ± SEM.



4.3.3 Rotarod

All animals improved at the task across training sessions, as indicated by a significant effect of session on latency to first fall (Main effect of SESSION: *Cdkn1c*^{BACx1} $F_{3.4,92.16}$= 29.056, p<0.001; *Cdkn1c*^{BACLacZ} $F_{4,112}$= 26.653, p<0.001) (Figure 4.3A,B). This improvement in task performance was not different by genotype (SESSION*GENOTYPE interaction: *Cdkn1c*^{BACx1} $F_{3.4,92.16}$= 1.805, p=0.14; *Cdkn1c*^{BACLacZ} $F_{4,112}$= 0.79, p=0.53), nor was there an effect of genotype on latency to fall (Main effect of GENOTYPE: *Cdkn1c*^{BACx1} $F_{1,27}$= 1.917, p=0.18, *Cdkn1c*^{BACLacZ} $F_{1,28}$= 0.248, p=0.62).

Figure 4.3: There was no effect of genotype on motor learning or motor co-ordination in a rotatrod task. A, B) All animal performance improved across sessions. There was no difference between *Cdkn1c*^{BACx1} animals and their wt littermates (A) or between *Cdkn1c*^{BACLacZ} animals and their wt littermates (B) in latency to fall. All data is displayed ± SEM. *** p<0.001

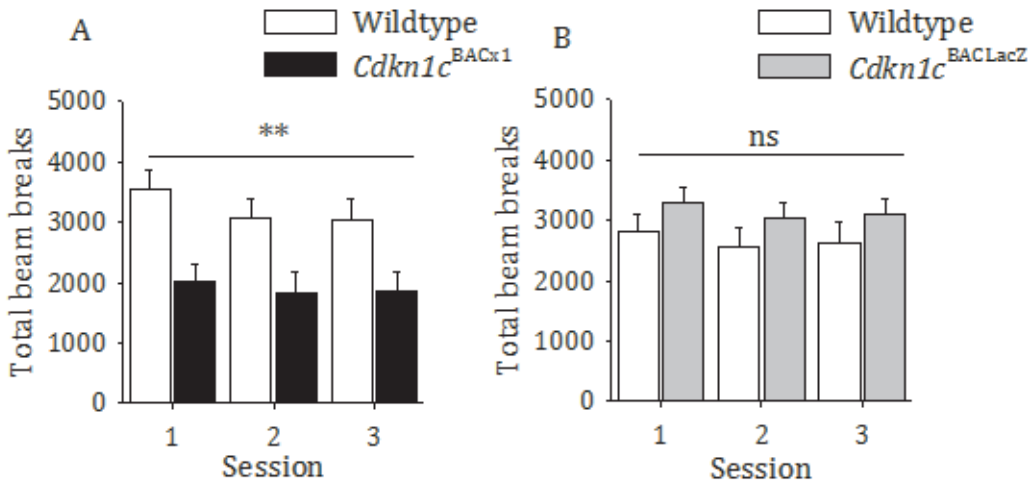


4.3.4 LMA

Across all three habituation trails, *Cdkn1c*^{BACx1} animals made significantly less beam breaks than their wt litter-mates (main effect of GENOTYPE: $F_{1,27}$=9.142, p<0.01) (Fig 4.4A). All animals habituated to the environment within each session as indicated by a decrease in beam breaks per bin within the first session (main effect of BIN: session 1, $F_{23,621}$=5.206, p<0.001), the rate of which was not different by genotype (BIN*GENOTYPE interaction: session 1, $F_{23,621}$=0.358, p=0.998). As well as this there was a strong trend for these animals to decrease in overall beam breaks made across the three sessions (main effect of SESSION: $F_{1.78,48.15}$=3.134, p=0.058), without a significant SESSION*GENOTYPE interaction ($F_{1.78,48.15}$=0.901, p=0.41), indicating that all animals habituated equally to the arena over time. Unlike, *Cdkn1c*^{BACx1} animals, *Cdkn1c*^{BACLacZ} animals did not differ from their wt littermates in the total number of beam breaks made across the three habituation sessions (main effect of GENOTYPE: $F_{1,28}$=1.459, p=0.24) (Figure 4.4B). As would be expected, all animals decreased the number of beam breaks made over time within the first

session as they habituated to the arena (main effect of BIN: session 1, $F_{23,644}$=9.875, p<0.001). Additionally, across sessions, *Cdkn1c*^{BACLacZ} animals and their wt littermates made fewer beam breaks per session (main effect of SESSION: $F_{1.94,54.26}$=3.285, p<0.05); and the rate of habituation across session was not different by genotype (SESSION*GENOTYPE interaction: $F_{1.94,54.26}$=0.001, p=0.999). Importantly there was no difference between wt animals of the separate lines in total number of beam breaks made per session (main effect of GENOTYPE: $F_{1,28}$=1.655, p=0.21), nor in the rate of habituation across sessions (SESSION*GENOTYPE interaction: $F_{1.82,51}$=0.564, p=0.57).

Figure 4.4: *Cdkn1c*^{BACx1} animals were hypoactive compared to their wt littermates. A) *Cdkn1c*^{BACx1} animals made significantly less beam breaks than their wt littermates across three sessions. B) This was not the case between *Cdkn1c*^{BACLacZ} animals and their wt littermates. All data is displayed ± SEM. ** p<0.01

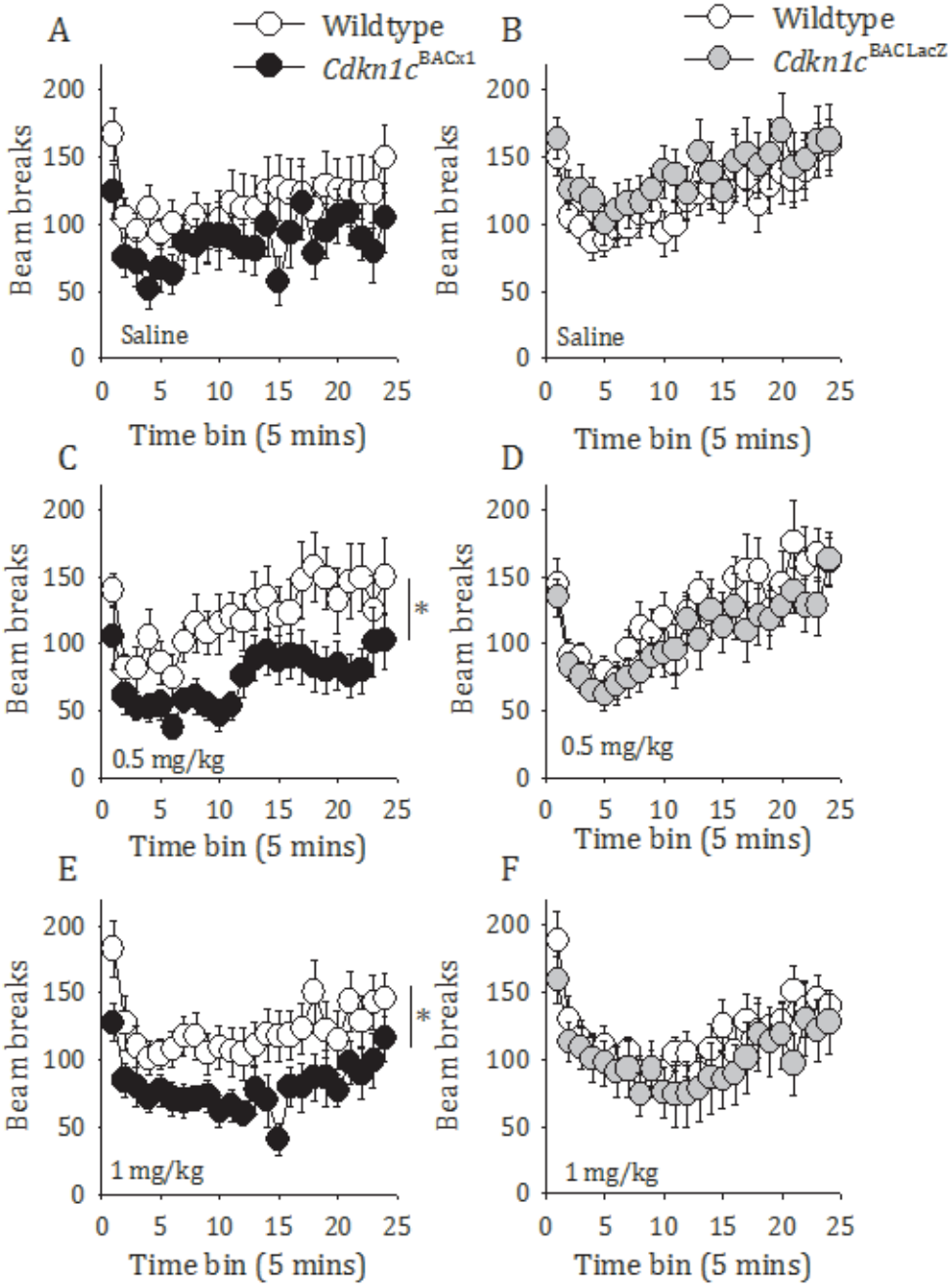


4.3.5 Amphetamine induced LMA

As expected from a low dose of amphetamine, there was no significant affect on locomotor activity over saline (Main effect of DRUG *Cdkn1c*^{BACx1}: $F_{1.7,46.2}$=0.053, p=0.927; *Cdkn1c*^{BACLacZ}: $F_{1.8,51.4}$=1.194, p=0.308). A repeated measures DRUG*BIN*GENOTYPE interaction for *Cdkn1c*^{BACx1} animals and their wt littermates displayed a main effect of genotype (Main effect of GENOTYPE: F1,27: 5.054, p=0.033). This contrasted with *Cdkn1c*^{BACLacZ} animals and their wt

littermates (Main effect of GENOTYPE: $F_{1,28}$: 0.118, p=0.73). Post-hoc tests, after Bonferroni adjustments for multiple correction, showed this to be as a result of *Cdkn1c*^{BACx1} animals moving significantly less than their wt littermates after injection of 0.5 mg/kg ($F_{1,28}$=4.619, p=0.041) or 1 mg/kg amphetamine ($F_{1,28}$=5.376, p=0.028) (Figure 4.5C,E), but not after saline injection ($F_{1,28}$=1.203, p=0.28) (Figure 4.5A). This was not the case between *Cdkn1c*^{BACLacZ} animals and their wt littermates, where there was no effect of genotype on the number of beam breaks made after injection of either saline ($F_{1,29}$=0.632, p=0.43) (Figure 4.5A), 0.5 mg/kg ($F_{1,29}$=0.838, p=0.37) (Figure 4.5D) and 1 mg/kg($F_{1,29}$=0.606, p=0.44) (Figure 4.5F).

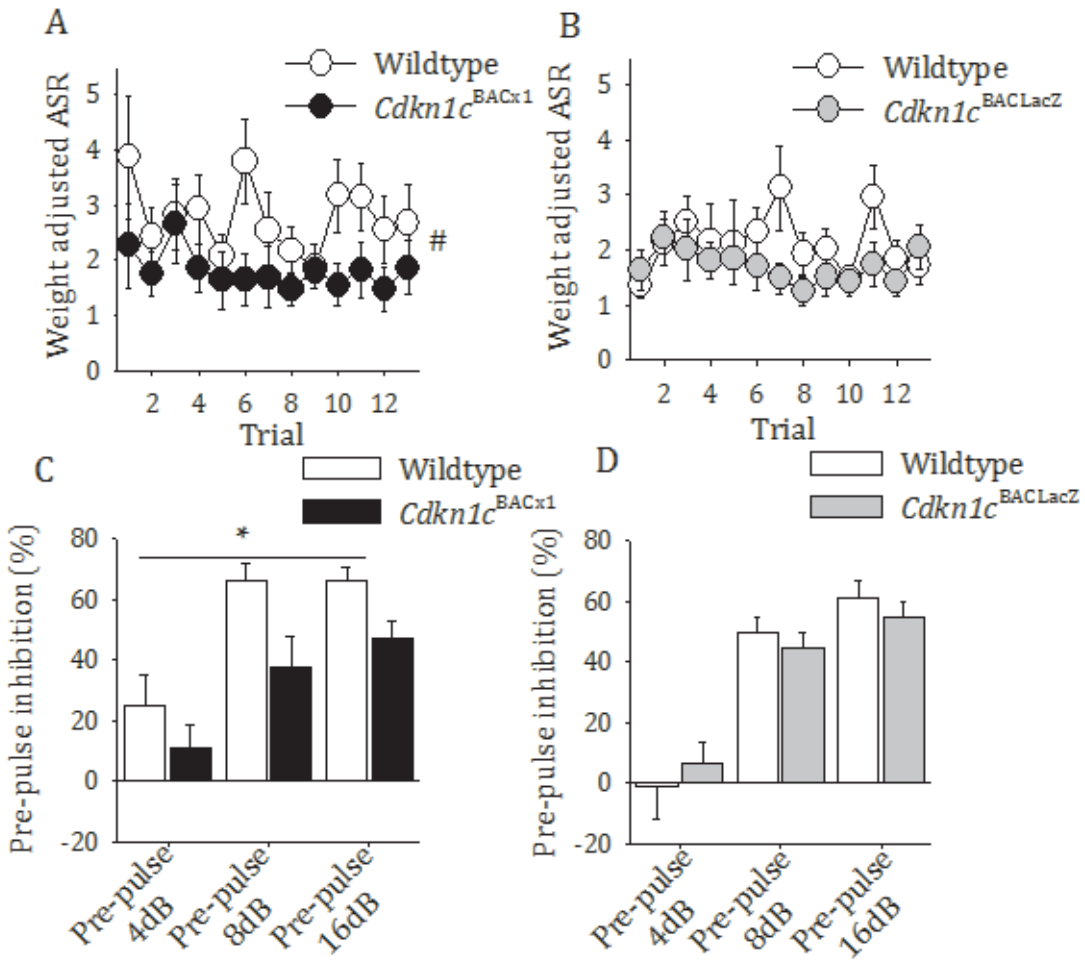
Figure 4.5: *Cdkn1c*^{BACx1} animals were hypersensitive to sub-stimulatory doses of amphetamine. A, B) There was no difference between *Cdkn1c*^{BACx1} animals and their wt littermates (A) or between *Cdkn1c*^{BACLacZ} animals and their wt littermates (B) in beam breaks after a saline injection. C, D, E, F) *Cdkn1c*^{BACx1} animals made significantly less beam breaks than their wt littermates after an injection of 0.5 mg/kg (C) or 1 mg/kg amphetamine (E) this was not the case between *Cdkn1c*^{BACLacZ} animals and their wt littermates (D, F). All data is displayed ± SEM. *p<0.05.



4.3.6 ASR and PPI

After adjusting for any differences in weight, there was no significant effect of genotype on the ASR at either 105 or 120 dB across the habituation trials (*Cdkn1c*^{BACx1} 120dB: $F_{1,29}$=1.214, p=0.28; 105dB: $F_{1,29}$=3.935, p=0.057; *Cdkn1c*^{BACLacZ} 120 dB: $F_{1,28}$= 0.094, p=760.05, 105dB: $F_{1,28}$= 1.558, p=0.22). *Cdkn1c*^{BACx1} animals had significantly blunted PPI at 105 dB (main effect of GENOTYPE: $F_{1,29}$=6.264, p=0.018) (Figure 4.6C), but not 120 dB (main effect of GENOTYPE: $F_{1,29}$=0, p=0.999). Post-hoc tests indicate that this is due to a difference in the effectiveness of the pre-pulse at 8 ($F_{1,30}$=5.873, p=0.022) and 16 dB ($F_{1,30}$=6.624, p=0.015), but not 4 dB ($F_{1,30}$=1.152, p=0.292), between *Cdkn1c*^{BACx1} animals and their wt littermates. It is unlikely that this phenomenon is related to auditory deficits in *Cdkn1c*^{BACx1} animals as the startle response to 105 dB is reduced with a pre-pulse in all animals, in an amplitude dependant manner (main effect of PRE-PULSE: $F_{1.8,52.09}$=22.926, p<0.001), as would be expected. *Cdkn1c*^{BACLacZ} startled to the same degree as their wt littermates after each pre-pulse at 105 dB ($F_{1,28}$=0.034, p=0.85) (Figure 4.6D) and 120 dB ($F_{1,28}$=2.837, p=0.1) and there was no difference between wt animals of the two strains in PPI at 105 dB ($F_{1,28}$=3.82, p=0.06) or 120 dB ($F_{1,28}$=1.512, p=0.23).

Figure 4.6. *Cdkn1c*^{BACx1} animals have sensorimotor gating deficits in a PPI task. A, B) There was a marginally non-significant difference between *Cdkn1c*^{BACx1} animals and their wt littermates (A) and no difference between *Cdkn1c*^{BACLacZ} animals and their wt littermates (B) in ASR to a 105 dB pulse across habituation trials. C, D) *Cdkn1c*^{BACx1} animals had a deficit in PPI to a 105 dB test pulse (C) this was not the case between *Cdkn1c*^{BACLacZ} animals and their wt littermates (D). All data is displayed ± SEM. *p<0.05 #p=0.057



4.4 Discussion

This chapter details the basic motoric and anxiolytic behavioural characterisation of the *Cdkn1c* over-expressing animals, *Cdkn1c*^{BACx1}, and their wt littermates, as well as transgenic animals not over-expressing *Cdkn1c,* *Cdkn1c*^{BACLacZ}, and their wt littermates. In addition, a number of tasks sensitive to dopamine levels were untaken. This followed on from the observation in

chapter 3 that over-expression of *Cdkn1c* leads to increased dopamine in the dorsal striatum and alterations in expression of genes related to dopaminergic function. No differences in anxiety or novelty reactivity were observed between genotypes of either line. Additionally motor learning in a rotarod task was intact in all animals. Over expression of *Cdkn1c* specifically resulted in hypoactivity, without a difference in the rate of habituation to a novel environment, implying the presence of a subtle basal locomotor phenotype. Additionally, the locomotion of *Cdkn1c*^{BACx1} animals was hypersensitive to sub-stimulatory doses of amphetamine, as shown by a decrease in the number of beam breaks compared to wt after an i.p. injection of an amphetamine solution. This phenomenon not observed in *Cdkn1c*^{BACLacZ} animals. Further data suggestive of altered dopaminergic function was the sensorimotor gating deficits observed in *Cdkn1c*^{BACx1} animals, as indexed by decreased PPI compared to wt. These findings indicated that gross motoric and anxiety related behaviours were intact after over expression of *Cdkn1c* but that, as predicted from the results in Chapter 3, phenotypes sensitive to levels of dopamine were altered in these animals.

4.4.1 EPM and OF

As expected all animals preferred the less anixogenic zones in the EPM and OF. Rodents' avoidance of open spaces is well documented (Lister, 1990; File, 2001). Willingness to explore the open arms or interior of an OF is indicative of a less anxious, or enhanced risk-taking state, and drugs that decrease anxiety in humans increase this propensity in rodents (Lister, 1987; Prut and Belzung, 2003; Ramos, 2008). Here, there was no significant effect of genotype, in either tine on the time spent in the anxiogenic zones or frequency of entry into anxiogenic zones. However, in the EPM and OF, *Cdkn1c*^{BACx1} animals made, non-significantly, fewer entries to, and spent less time in, the anxiogenic zones. This suggests of a potential anxious phenotype. However, there were no differences in the rate of habituation to a novel OF or in the total distance moved. Therefore, habituation to novel areas prior to testing, unless part of testing protocol, was used in Chapters 5 and 6 to account for any difference in anxiety.

4.4.2 Motoric function

Rotarod tasks are routinely used to assess motor coordination and motor learning in rodents, especially when deficits in the midbrain dopaminergic system are expected or induced (Carter et al., 1999; Paumier et al., 2013; Wang et al., 2013; Pang et al., 2014). Given that *Cdkn1c*^{BACx1} animals have alterations in this region, as shown in Chapter 3, a rotarod task was carried out. As expected, across sessions, the latency to fall from the rotarod increased, implying that all animals learned to perform the task and stay on the rotating rod. Importantly, there was no effect of genotype in either line on the latency to fall implying that motor co-ordination was equivalent between groups.

LMA, as assayed by number of beam breaks, as opposed to distance travelled in an OF, provides similar but distinct information about the activity levels of an animal. *Cdkn1c*^{BACx1} animals were shown here to be hypoactive across the three habituation trials, compared to their wt littermates, a finding not observed between *Cdkn1c*^{BACLacZ} animals and their wt littermates. As expected, all animals habituated to the arena within a session as indicated by a decrease in beam breaks across the session. Additionally, all animals habituated to the arena across sessions as indicated by a decrease in total beam breaks across the three habituation sessions. This decrease was marginally non-significant for *Cdkn1c*^{BACx1} animals and their wt littermates, potentially as a result of the observed hypoactivity of *Cdkn1c*^{BACx1} animals resulting in a 'floor effect' for the group. Importantly, there was no difference between the wt animals of either strain highlighting that this difference was specifically as a result of *Cdkn1c* over expression. As there was no effect of genotype on the rate of habituation, it is likely *Cdkn1c*^{BACx1} hypoactivity was not as result of differential learning about the novel arena or as a result of enhanced fearfulness of novelty.

The seeming disparity between levels of activity between distance moved in an OF and beam breaks in a novel arena is intriguing. This could indicate a very subtle motoric phenotype, potentially relating to increased stereotyping behaviours, i.e. repeated crossing of the same beam. However, *Cdkn1c* animals made less 'same' breaks as well as making fewer total beam breaks (data not

shown). Alternatively, this phenotype may relate to darting behaviours from side of the arena to the other, with longer inter-movement pauses. However, this does not seem to be the case as there was no difference in total time spent moving in the OF field task (data not shown).

A number of genetically hyperdopaminergic animals, as a result of knockdown of *Dat*, display hyperactivity (Ralph et al., 2001; Zhuang et al., 2001; Napolitano et al., 2010; O'Neill and Gu, 2013) but this was not observed with *Cdkn1c*^{BACx1} animals. However, the increases in dopamine after DAT knock down are several fold greater (Zhuang et al., 2001) than are observed in *Cdkn1c*^{BACx1} animals, as shown in Figure 3.3B.

Injection of 0.5 mg/kg or 1 mg/kg of amphetamine had no effect on locomotor activity, either of wt animals or of *Cdkn1c*^{BACLacZ} animals. These data must be interpreted with caution in the absence of a drug effect on wt animals. However, both doses resulted in a decrease in locomotor activity of *Cdkn1c*^{BACx1} animals compared to their wt littermates. This is not without precedent. The paradoxical calming affects of stimulants are well documented in the case of genetically hyper-dopaminergic animals, specifically as a result of *Dat* knockdown/out (Gainetdinov, 1999; Zhuang et al., 2001; Napolitano et al., 2010). This has clinical relevance with respect to attention deficit hyperactivity disorder (ADHD), in which stimulants of the dopaminergic system are used as a successful means of treatment. Given the observation in chapter 3 (Figure 3.7) that cells in the nucleus accumbens of *Cdkn1c*^{BACx1} animals are hypersensitive to amphetamine; it is not unexpected that this sub-stimulatory dose produces such a profound motoric effect.

It is possible that the observed difference in locomotor activity occured as a result of the interaction between the observed hypoactivity and an increased locomotor response to an injection of saline, masking this difference for the saline trial alone. While this is a potential explanation, it is unlikely as injection order was counterbalanced for solution and genotype, such that an animal's first injection was not necessarily saline. Therefore, the observed absence of

genotype effect after a saline injection was not due to a heightened response of *Cdkn1c*^{BACx1} animals to a novel noxious stimulus.

4.4.3 Acoustic startle and PPI

Unexpectedly, no habituation, indexed by a decrease in startle amplitude over time, was observed in any animals across the trials. Given that this occurred in all genotypes it is unlikely to be as result of genetic manipulation or environmental factors in the home cage influencing behaviour. It is possible that this is due to environmental factors within the apparatus or testing room. There was a marginally non-significant trend (p=0.057) for *Cdkn1c*^{BACx1} animals to startle less than their wt littermates to 105 dB acoustic pulse across habituation trials, an observation not seen in *Cdkn1c*^{BACLacZ} animals. This has a potential for influencing the PPI measure (Sandner and Canal, 2007). However, it is important to note that PPI is a measure of percentage difference from baseline and, in this way, accounts for any baseline differences. These observations are unlikely to be linked to an acoustic deficit in *Cdkn1c*^{BACx1} animals as there was the expected significant effect of pre-pulse volume on all animals PPI, with louder pre-pulses inhibiting the ASR to a greater degree. Perhaps unexpectedly, there was no effect of genotype on ASR or PPI when animals were presented with a 120 dB startling pulse. This is likely to be as a result of the greater startling capacity of a 120 dB pulse overall, masking any differences between the groups. Additionally these observations were not due to an altered anxiety state of *Cdkn1c*^{BACx1}, as no differences were observed in an OF or EPM test. This highlights the specificity of the deficit in sensorimotor gating in these animals over-expressing *Cdkn1c*.

4.4.4 Conclusions

Over expression of *Cdkn1c* caused subtle, very specific alterations in some basic behaviours. Animals over-expressing *Cdkn1c* were hypoactive compared to their wt littermates with respect to beam breaks, but not total distance moved in an open field. This was without any differences in motor co-ordination or motor learning. Additionally, as inferred from chapter 3, these animals were hypersensitive to a sub-stimulatory dose of amphetamine. This resulted in a counter-intuitive, but not unexpected, blunting of locomotor activity. Over

expression of *Cdkn1c* also caused subtle deficits in sensorimotor gating, with a reduced PPI when presented with a 105 dB acoustic pulse, but not 120 dB. This occurred without any significant differences in anxiety levels in two separate assays of anxious behaviours. Overall, these results highlight the presence of an altered dopaminergic state of animals over-expressing *Cdkn1c*, but do not present any significant confounds that could interfere with further behavioural testing.

Chapter 5: *Cdkn1c* and reward

5.1 Introduction

Dopamine has been long associated with reward processing through evidence from drug and genetic studies in humans and model organisms. It is believed to have a role in the incentive salience of, but not necessarily the hedonistic response to, a reinforcer (Berridge et al., 2009). Pharmacologically and genetically induced hyper-dopaminergic states in rodents produce an increase in the incentive salience of a reinforcer (Wyvell and Berridge, 2001; Covelo et al., 2012; Bauer et al., 2013; Peciña and Berridge, 2013). Increasing dopamine in rodents, either by administering amphetamine or by knock-down of *Dat*, increases an animal's tendency to press a lever associated with a reward over a control lever as well as resulting in decreased latency to reward collection in a runway task (Wyvell and Berridge, 2000; Pecina et al., 2003). Dopamine deficient mice do not learn to press a lever for a reward, whereas control mice will. This deficiency can be reversed by restoring dopamine either by giving the animals L-DOPA or by restoring dopamine synthesis in the midbrain only (Robinson et al., 2007). Conversely, antagonists of the DAergic system produce a reduction in a reinforcer's incentive salience (Dickinson et al., 2000; Lex and Hauber, 2008; Ostlund and Maidment, 2012) (Pielock et al., 2011; Wassum et al., 2011; Saunders and Robinson, 2012). Additionally, both predicted and unpredicted sucrose pellets evoke an increase in DA in striatal subregions (Brown et al., 2011) and food rewards also increase accumbal DA (Hernandez and Hoebel, 1988a, b; Joseph and Hodges, 1990). Finally, drugs of abuse in humans, such as cocaine and amphetamine, have their site of action directly on DAergic neurons. Together, these results highlight the specific importance of midbrain dopamine in motivation to work for a reward.

The first task described in this chapter is the lick microstructure analysis task. Hedonic response is most commonly assessed by consumption or preference in a two-bottle choice test (Hajnal et al., 2004; Drew et al., 2007; Romieu et al., 2008; Vucetic et al., 2010b; van der Plasse et al., 2012; Carlin et al., 2013). This is informative, however is confounded by the interaction between consumption

(and number of licks) and increasing solution concentration (Spector et al., 1998). Another method used to infer perceived palatability of a solution is analysis positive affective orofacial movements during consumption (Pecina et al., 2003; Mahler et al., 2007; Faure et al., 2010; Shin et al., 2011). This reliably reports an animal's hedonic response to a palatable solution but is technically challenging and, to a degree, subjective data to analyse. Lick microstructure analysis produces a quantitative measure which represents an animal's hedonic response to a solution, independent of consumption volume. Rodents will consume freely available palatable liquids, such as sucrose, in a predictable manner (Davis, 1998; Dotson and Spector, 2005; Boughter et al., 2007; Johnson et al., 2010a; Lin et al., 2013). The pattern of licks made is in a series of 'bursts' or 'clusters', the size of which is determined by the palatability of the solution, with lick number per cluster increasing linearly with increasing concentration of a palatable solution (Dwyer et al., 2009; Yoneda et al., 2009; Lydall et al., 2010; Wright et al., 2013). Therefore, the hedonic state of an animal can be probed reliably using this task.

The second task in this chapter concerns an animal's motivation to work for a reward, i.e. the incentive salience of the reinforcer, as indexed by break point (BP) in a progressive ratio (PR) task. Mice will learn to nose poke to obtain a reward and in a PR task the number of nose poke required to obtain a reward increases over trials within a single session. Essentially, this means, within a session, an animal must work increasingly harder for the same reward. The maximum number of nose pokes it is willing to make to receive the reward is termed the "break-point" and this has been shown to be sensitive to manipulations of the midbrain dopaminergic system (Salamone et al., 2001; Thomsen et al., 2009; Covelo et al., 2012; Skibicka et al., 2013). Restriction of dopamine signalling to dorsal striatum is sufficient for mice to perform a PR task (Darvas and Palmiter, 2009) highlighting the importance of this area in motivation to work for a reinforcer. Amphetamine microinjection to the nucleus accumbens also increases the BP in a PR task (Zhang et al., 2003) implying a significant role for midbrain dopaminergic activity in the performance of this task.

As shown in Chapter 3, animals over-expressing *Cdkn1c* have elevated DA in the dorsal striatum, as well as increased *Th* expression in the striatum with elevated TH immuno-reactivity in the same region. Additionally, as shown in Chapter 4, these animals have changes in sensorimotor gating as well as hypersensitivity to amphetamine in the striatum, phenomena both associated with a neural hyper-dopaminergic state. Therefore, given the link between DA and reward processing, this chapter focuses on the motivational state of *Cdkn1c*^{BACx1} animals in comparison to their wt littermates. The hedonic state of *Cdkn1c*^{BACx1} and *Cdkn1c*^{BACLacZ} animals, as well as their wt littermates was assessed using lick microstructure analysis. Following this, the incentive salience of a reinforcing sucrose solution was assessed used PR task in a subset of animals.

5.2 Methods

5.2.1 Animals

109 male mice were used for the licking microstructure analysis, genotype: *Cdkn1c*^{BACx1} (n=29), wt littermates (n=27), *Cdkn1c*^{BACLacZ} (n=31) wt littermates (n=22). A subset of these were used for PR testing genotype: *Cdkn1c*^{BACx1} (n=14) wt littermates (n=13), *Cdkn1c*^{BACLacZ} (n=17), wt littermates (n=9). All animals were between 7 and 9 weeks at the start of lick cluster analysis testing and between 13 and 15 weeks at the start of PR testing. General housing and husbandry conditions were as described in Chapter 2.1.

5.2.2 Licking micro-structure analysis

Mice were on restricted food access for the duration of testing, having no access to food for 16 hours prior to testing. Testing was carried out as described in Lydall *et al*. (Lydall et al., 2010) but adapted for mice. Briefly, testing took place in white Perspex boxes (32× 15×12 cm) with metal grid floors and wire mesh lids. Access to the solution was provided, in 50 ml bottles attached to stainless steel drinking spouts, for thirty minutes, once per day. Testing was conducted between 08:00 and 11:00. A contact sensitive lickometer registered each lick to

the nearest 0.01 s, recorded using MED-PC software (Med Associates Inc., St. Albans, VT, USA). Consumption for each session was recorded by weighing bottles before and after each session. Assuming 1 ml of solution ≈ 1 g, consumption was then normalised to weight that day (consumption (ml)/(body weight (g) $^{0.75}$)), to account for any differences in size between the groups (Doe et al., 2009; Relkovic et al., 2010). A range of sucrose concentrations were used (2, 4, 8 and 16% w/w) (approximately 0.06, 0.12, 0.24 and 0.48 M) and dissolved in deionised water. Animals were trained to drink 8% sucrose prior to testing, until consumption had stabilised (requiring 4-14 sessions). Animals were presented with solution of a given concentration for a minimum of four days; the order in which animals were presented with each concentration was counterbalanced for ascending or descending concentration presentation and genotype. Sessions in which an animal's behaviour was extremely different to the group normal were excluded; specifically making less than 10 bouts, greater than 40 one lick bouts or having an inter-lick interval of <90 or >250 ms. Behaviour for a minimum of four sessions was examined at each concentration and data from the first day at each new concentration was excluded from analysis to eliminate any effects of neophobia (Bahar et al., 2004; Figueroa-Guzmán and Reilly, 2008; Pedroza-Llinas et al., 2009). Data was averaged across sessions at each concentration for comparison. A pause between licks of greater than 0.5 s was defined as a new cluster.

5.5.3 PR

For the duration of testing animals were on a restricted water access schedule, water provided for two hours immediately after testing, testing taking place between 08:00 and 11:00. Testing took place in a 9-hole box modified for use in mice, with four alternate holes in the horizontal array covered and the central hole was illuminated. Mice were initially trained for three days to press a Perspex panel opposite the array of holes in order to gain access to reinforcer, in this case 8% (w/w) sucrose (approximately 0.24 M), as above. During testing a nose poke by the mouse in the illuminated hole resulted in the delivery of 20 µl of reinforcer behind the Perspex panel and collection of this reward initiated a

subsequent trial. Continuous reinforcement (CRF) (i.e. one nose poke required for reward delivery) was carried out for four days. Following this, a PR schedule was carried out whereby within a session animals had to nose poke an increasingly higher number of times for reinforcer delivery. Number of nose pokes required ascended linearly every four trials (FR4) for three sessions, followed every two trials (FR2) a further three sessions. This was followed by four CRF sessions. A single probe trial at the FR2 PR schedule was carried out using 2% (w/w) sucrose, followed by a day of CRF. An additional single probe trial at FR2 was carried out when animals were allowed *ad libitum* water access for 24 hours prior to testing.

To determine whether animals were responding to the calorifically rewarding aspect of sucrose animals were testing using an iso-sweet to 8% sucrose concentration of saccharin (0.1% (w/w)) (approximately 6.6 mM). Animals were trained to obtain a reward of 0.1% saccharin in a CRF schedule for two days. Following this a probe trial at FR2 was carried out with 0.1% saccharin.

5.2.4 Statistical analysis

All statistical analyses were carried out using SPSS 20.0 (SPSS, USA). A series of ANOVAs were carried out on parameters below after ensuring normality of data. For lick analysis a repeated measures ANOVA was carried out, a between subject factor of GENOTYPE and within subjects factor of CONCENTRATION. For PR analysis a reaped measures ANOVA was carried out with a between subjects factor of GENOTYPE and the within subjects factor was SESSION. Bonferroni post-hoc tests were carried out where appropriate.

5.3 Results

5.3.1 Lick micro-structure analysis

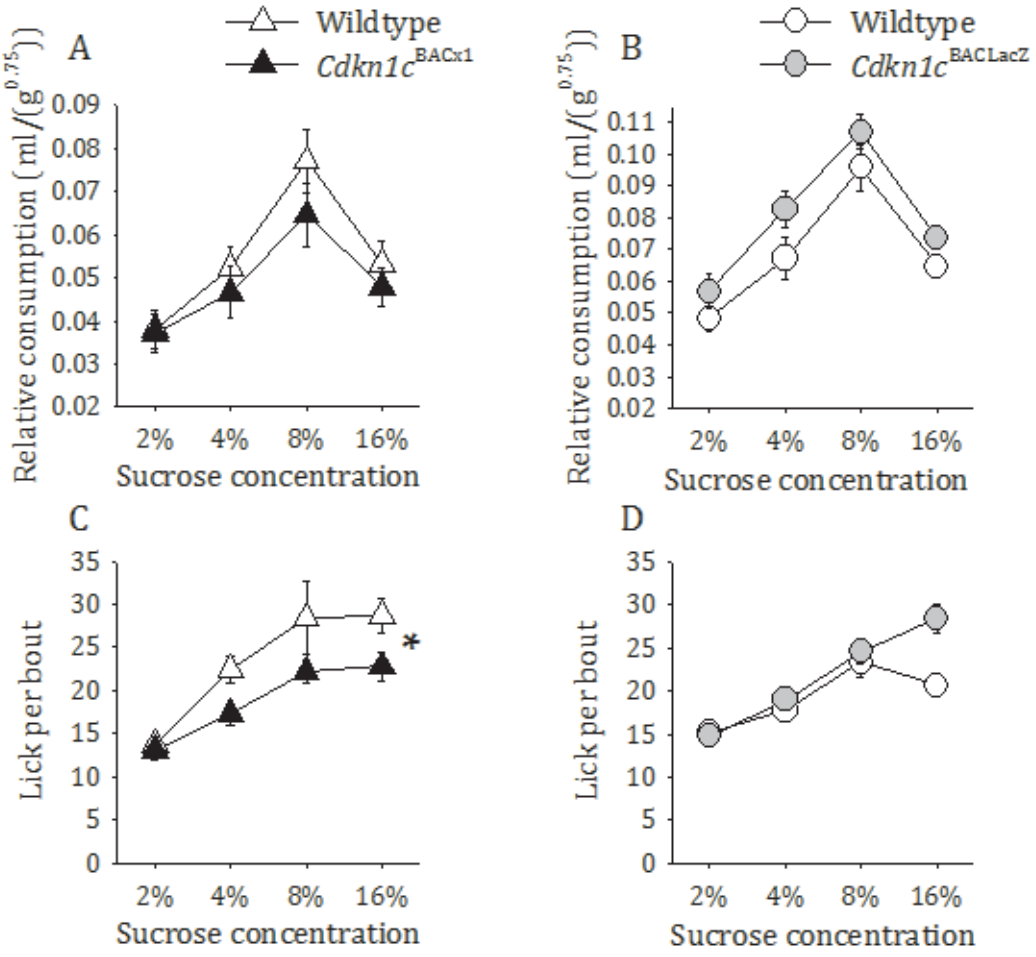
During training animals consumed freely available 8% sucrose solution for 30 mins after 16 hours food deprivation. Individuals were moved on to the test phase after consumption volume had stabilised for a minimum of 2 days.

Animals were then presented with one of 2, 4, 8 or 16% sucrose, each for a minimum of four days.

Sucrose consumption formed an inverted U-shaped curve, peaking at 8% sucrose, as expected. There was a significant effect of concentration of sucrose solution on consumption (main effect of SOLUTION: *Cdkn1c*^{BACx1}: $F_{3,162}$=25.572, p<0.001; *Cdkn1c*^{BACLacZ}: $F_{3,153}$=40.18, p<0.001), without an effect of genotype in the case of *Cdkn1c*^{BACx1} and their wt littermates (main effect of GENOTYPE: $F1_{,54}$=0.884, p=0.351) (Figure 5.1A). There was a marginally significant difference in consumption between *Cdkn1c*^{BACLacZ} and their wt littermates (main effect of GENOTYPE: $F_{1,51}$=4.197, p=0.05) (Figure 5.1B).

As concentration and therefore palatability of the sucrose solution increased there was a significant increase in the average lick cluster size for both strains (main effect of SOLUTION: *Cdkn1c*^{BACx1}: $F_{3,162}$=27.094, p<0.001; *Cdkn1c*^{BACLacZ}: $F_{3,153}$=44.962, p<0.001). Interestingly, *Cdkn1c*^{BACx1} had significantly smaller average lick cluster sizes compared to their wt littermates ($F_{1,54}$=5.109, p<0.05) (Figure 5.1C). This was not observed between *Cdkn1c*^{BACLacZ} and their wt littermates ($F_{1,51}$=2.938, p=0.1) (Figure 5.1D).

Figure 5.1 Animals over-expressing *Cdkn1c* found sucrose solutions less palatable than wt. A, B) Relative consumption of sucrose across four concentrations for *Cdkn1c*^{BACx1} animals and their wt littermates (A) and *Cdkn1c*^{BACLacZ} and their wt littermates (B). C, D) Average lick cluster size at four concentrations of sucrose for *Cdkn1c*^{BACx1} and wt littermates (C) and *Cdkn1c*^{BACLacZ} and wt littermates (D). Data shown ±SEM. *p<0.05



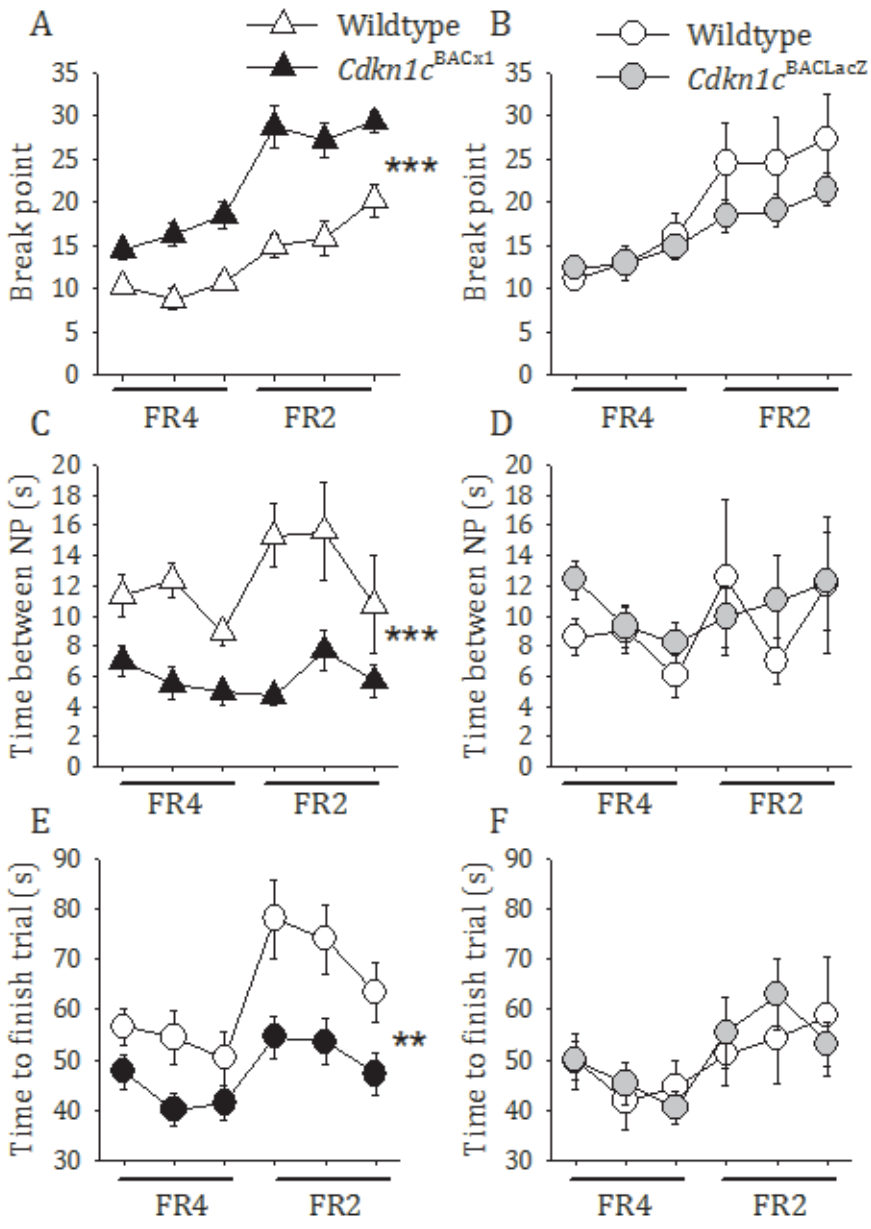
5.3.2 PR

A subset of animals was then tested in a PR task to assess motivation to work for a reinforcer, in this case 8% sucrose. This concentration was chosen as it was found, in lick cluster analysis, to be the concentration at which all animals consumed maximally. All animals acquired the task rapidly in the CRF condition, completing an average of 71.88 (SEM 4.2) (*Cdkn1c*^{BACx1}) and 82.52 (SEM 2.7) (*Cdkn1c*^{BACLacZ}) trials within 4 days of testing. Trials completed was not different

between genotypes in the CRF condition (Main effect of GENOTYPE: *Cdkn1c*^{BACx1}: $F_{1,23}$=2.51, p=0.127; *Cdkn1c*^{BACLacZ}: $F_{1,23}$=0.083, p=776).

In a PR schedule, when the number of nose-pokes required to receive a reward increased within a session, *Cdkn1c*^{BACx1} had a significantly higher BP than their wt littermates ($F_{1,23}$=17.109, p<0.001) (Figure 5.2A). This finding was not observed between *Cdkn1c*^{BACLacZ} animals and their wt littermates ($F_{1,23}$=1.012, p=0.33) (Figure 5.2B). In addition to having a higher BP, *Cdkn1c*^{BACx1} animals had a shorter inter nose-poke interval ($F_{1,23}$=28.56, p<0.001) (Figure 5.2C) and were quicker to complete trials ($F_{1,23}$=12.442, p<0.01) (Figure 5.2E). This was not the case between *Cdkn1c*^{BACLacZ} and their wt littermates (inter nose-poke interval: $F_{1,23}$=0.236, p=0.61 (Figure 5.2D); time to complete trial $F_{1,23}$=0.036, p=0.85 (Figure 5.2F)).

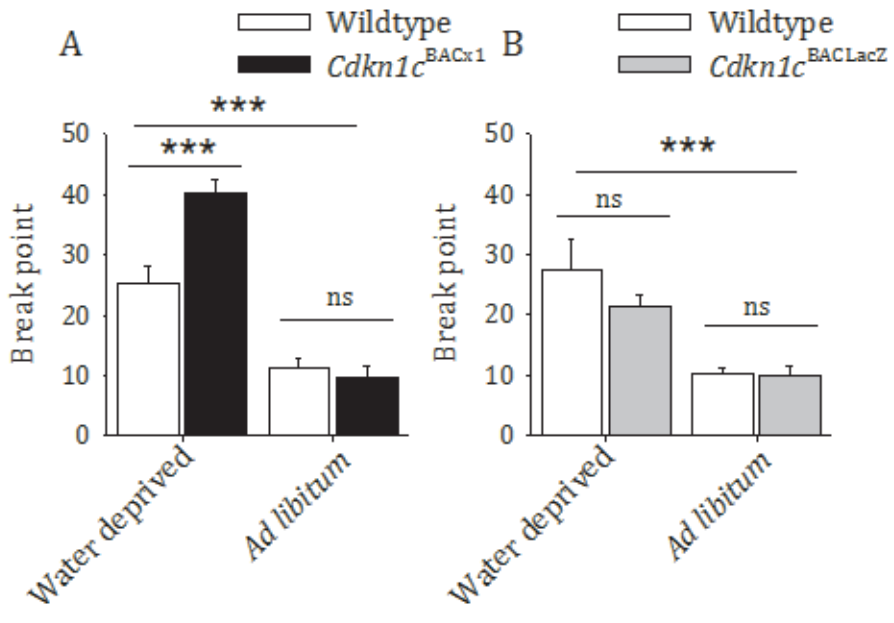
Figure 5.2. *Cdkn1c*^{BACx1} animals more motivated to obtain a sucrose reward compared to wt. A, B)BP in a PR task, C, D) average inter nose-poke interval and E, F) average time to complete a trial for *Cdkn1c*^{BACx1} animals (A, C, E) and their wt littermates and *Cdkn1c*^{BACLacZ} animals and their wt littermates (B, D, F). Data shown ±SEM **p<0.01 ***p<0.001



In order to demonstrate the observed differences in BP were due to differences in goal-directed responding and not as a product of over-training or habit formation, a single probe trail was carried out on a FR2 schedule in which the animals had *ad libitum* access to water for 24 hours prior to testing. As

expected, without the appetitive drive to obtain a sucrose reward, the BP of all animals was significantly lower (*Cdkn1c*^{BACx1}: $F_{1,23}$=23.156, p<0.001 (Figure 5.3A), *Cdkn1c*^{BACLacZ}: $F_{1,23}$=28.601, p<0.001 (Figure 5.3B)). A significant interaction term for BP between water restricted and *ad libitum* water fed states for *Cdkn1c*^{BACx1} animals and their wt littermates ($F_{1,23}$=15.325, p<0.001) was observed. Bonferroni post-hoc test revealed this to be as a result of a significant difference in BP when motivated ($F_{1,23}$=14.451, p<0.005) compared to in an *ad libitum* water fed state ($F_{1,23}$=0.41, p=0.53). Conversely, though *ad libitum* water access prior to testing reduced *Cdkn1c*^{BACLacZ} and their wt littermate's BP, there was no difference between genotypes in either state (water restricted: $F_{1,24}$= 0.098, p=0.76; *ad libitum* access: $F_{1,24}$= 0.029, p=0.87). This shows that PR responding was goal-directed in both groups.

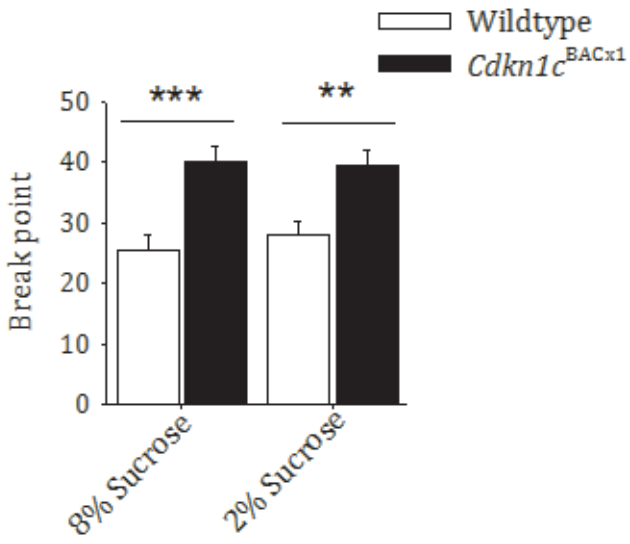
Figure 5.3. Responding in a PR task was goal directed. BP was significant reduced when unmotivated to work for both *Cdkn1c*^{BACx1} animals and their wt littermates (A) and *Cdkn1c*^{BACLacZ} animals and their wt littermates (B). Data shown ±SEM. ***p<0.005



Given that *Cdkn1c*^{BACx1} had a decreased perceived palatability of 8% sucrose compared to their wt littermates it was necessary to determine that the observed differences in BP were not due a decreased hedonic effect of the

solution, increasing the motivational drive. As shown in Figure 5.1C, there was no difference between *Cdkn1c*^{BACx1} and their wt littermates in the average lick cluster size, and therefore, perceived palatability of a 2% sucrose solution. However when the effort required to receive a reward of 2% sucrose increased within a PR session, animals over-expressing *Cdkn1c* continued to work harder to receive the reward, as indexed by a significantly higher BP ($F_{1,24}$= 11.118, p=0.003) (Figure 5.4), despite perceiving it to be as palatable as wt animals and consuming no more of it when freely available (Figure 5.1A).

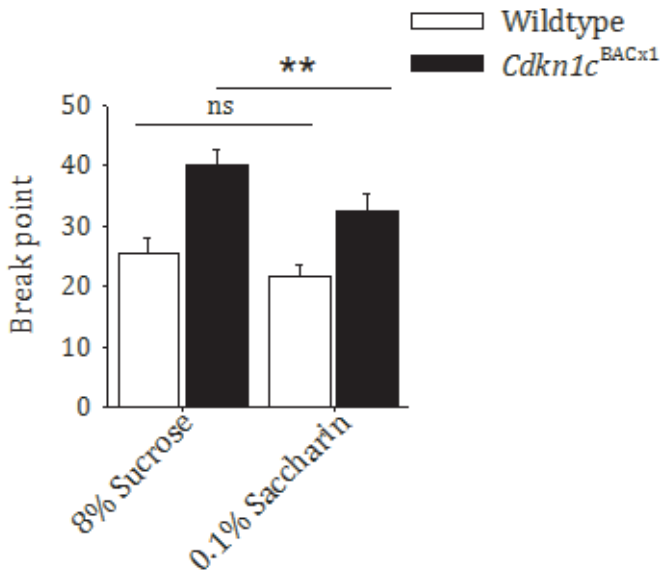
Figure 5.4 Animals over-expressing *Cdkn1c* maintained a significantly higher BP than their wt littermates even at concentrations of solution that were equally palatable. Average BP in a PR task when working for either an 8% or 2% sucrose solution. Data shown ±SEM **p<0.01 ***p<0.001



To differentiate between the calorific and hedonistic rewarding properties of sucrose, the animals' motivation to work for an iso-sweet solution of calorie-free, saccharin (0.1% (w/w) saccharin) was assessed at an FR2 schedule. Animals were first trained to consume saccharin on a CRF schedule for two days with *Cdkn1c*^{BACx1} wt and *Cdkn1c*^{BACx1} animals completing an average of 67.5 (SEM 5.2) and 76.14 (SEM 4.6) trials, respectively. All animals were less motivated to work for saccharin as compared to sucrose, as indexed by a decrease in BP (main effect of SOLUTION $F_{1,23}$=10.293, p=0.004. Bonferroni

post-hoc tests showed this to be driven by the significant decrease in BP of *Cdkn1c*^{BACx1} animals ($F_{1,23}$= 10.578, p=0.004) compared to a non significant decrease by their wt littermates ($F_{1,23}$= 1.972, p=0.174) (Figure 5.5). This implies that animals over-expressing *Cdkn1c* were more motivated than their wt littermates by the calorific rewarding properties of sucrose.

Figure 5.5 *Cdkn1c*^{BACx1} were more motivated to the calorific reward of sucrose, rather than the hedonic sweetness. Average BP in a PR task when working to obtain 8% sucrose or an iso-sweet concentration (0.1%) of the calorie free sweetener saccharin. Data shown ±SEM **p<0.01



5.4 Discussion

In this chapter the reward processing of animals over-expressing *Cdkn1c*^{BACx1} was assessed compared to their wt littermates. In addition, we examined the same functionality in transgenic animals not over-expressing *Cdkn1c*. All animals acquired the task at equivalent rates, implying that that operant learning was intact. Interestingly, animals over-expressing *Cdkn1c* appeared to have a hedonic deficit when consuming a naturally rewarding sucrose solution. However, when required to work to obtain the same rewarding solution these animals displayed an increased motivational drive. This was the case even at sucrose concentrations that were equally palatable between groups and was

goal-directed in nature, with a particular emphasis placed on the calorifically rewarding properties of sucrose by *Cdkn1c*^{BACx1} animals.

5.4.1 Hedonic response to a rewarding solution

All animals displayed the expected pattern of behaviour in response to a freely available palatable solution. As in previous experiments using these general methods and equipment (Lydall et al., 2010; Dwyer et al., 2011), a cluster was defined as a series of licks, with each lick separated by no more than a 0.5-s interval. The same criterion had been adopted by Davis and his colleagues (Davis, 1989; Davis and Smith, 1992; Davis and Perez, 1993). Although other criteria have been used (e.g., 1 s by (Spector et al., 1998)), there is little practical difference, since most pauses greater than 0.5 s are also greater than 1 s (Davis and Smith, 1992; Spector et al., 1998). Spector and colleagues (Spector et al., 1998) found that a food deprived state did not affect lick cluster size when the pause criterion was 1s but did have an effect when the pause criterion was 0.3 s, therefore an inter-cluster interval of >0.3s was chosen. Consumption formed an inverted U shaped curve, with maximal consumption at 8% sucrose. For all animals, average lick cluster size increased as the concentration of the sucrose solution increased. As perceived palatability increased to 8%, consumption rose, dipping slightly at 16% sucrose, likely reflecting increased calorific load of the solution and therefore more rapid satiety. Interestingly animals over-expressing *Cdkn1c* had significantly smaller average lick cluster sizes, compared to their wt littermates, which was not the case for animals carrying a transgene that did not over-express *Cdkn1c*. This may have clinical relevance as children with SRS, the human syndrome in which *CDKN1C* may be over expressed, are reportedly fussy eaters (Blissett et al., 2001). This implies these children may have a decreased perceived palatability of food as a consequence of increased *Cdkn1c.*

5.4.2 Motivation to obtain a natural reward as assayed in a PR task

In the PR task, all animals acquired the task, learning to nose poke to obtain a reward in the CRF schedule, regardless of genotype. CRF schedule performance has been shown to be insensitive to accumbal dopamine depletions (Aberman and Salamone, 1999), and is instead, dependent upon appetitive state of the individual. Linear, rather than exponential, ascension in the PR schedule was

chosen as mice complete far fewer trails in an exponential ascension paradigm (Finger et al., 2010), limiting analysis. As expected, all animals received less rewards when the effort required in obtaining a reward increased, in the FR4 and FR2 schedules. However, animals over-expressing *Cdkn1c* had a significantly higher BP than their wt littermates at both FR4 and FR2 PR schedules implying that these animals were willing to work harder than their wt littermates to obtain a reward of 8% sucrose. This was not the case for *Cdkn1c*$^{\text{BACLacZ}}$ animals with respect to their wt littermates. As well as being more motivated to obtain the reinforcer, *Cdkn1c*$^{\text{BACx1}}$ animals were more directed in obtaining the reinforcer, having shorter inter nose-poke intervals and a shorter latency to complete a trial compared to their wt littermates. Again, this was not the case for *Cdkn1c*$^{\text{BACLacZ}}$ animals and their wt littermates.

Crucially, there was no significant difference in the performance of wt animals between the two strains. Equally important is the observation that the performance of *Cdkn1c*$^{\text{BACx1}}$ animals was significantly different from that of *Cdkn1c*$^{\text{BACLacZ}}$ animals, allowing attribution of the phenotype specifically to over-expression of *Cdkn1c.* Additionally, all animals' responding was goal directed, as when not motivated to obtain the reinforcer (i.e. when allowed *ad libitum* water access prior to testing), all animals had a significantly reduced BP and the group differences in responding were abolished.

The performance of animals over-expressing *Cdkn1c* was significantly driven by the calorifically rewarding properties of sucrose, as shown by a decrease in BP of *Cdkn1c*$^{\text{BACx1}}$ animals when working to obtain an iso-sweet concentration of the calorie free sweetener, saccharin. This is aligned with the findings from the lick microstructure analysis which showed that *Cdkn1c*$^{\text{BACx1}}$ animals have a decreased perceived palatability of a sweet solution. Therefore, without the calorific motivational aspect of sucrose, these animals' BP is significantly reduced in a PR task, unlike wt animals. Importantly, this is not due to a specific sensitivity or aversion of these animals to saccharin as all animals displayed a significant preference for sucrose over water and also saccharin over water (data not shown). Additionally, all animals preferred sucrose over saccharin as

has been observed previously (Collier and Novell, 1967; Smith and Sclafani, 2002). These imply that while all animals find saccharin hedonically rewarding over water, the calorifically rewarding properties of sucrose are regarded more favourably.

5.4.3 Conclusions

Over expression of *Cdkn1c* resulted in animals with a decreased perceived palatability of a palatable sucrose solution. These animals also worked harder to obtain a sucrose reward, including at concentrations of sucrose that were perceived to be equally palatable. This increased motivation appeared to be as a result of an increased sensitivity of *Cdkn1c*$^{\text{BACx1}}$ animals to the calorifically rewarding properties of the sucrose, as these animals' BP was more affected by the switch to working for a calorie free, iso-sweet, saccharin solution. Taken together, the results from this series of experiments imply that *Cdkn1c* over-expression causes animals to work harder to receive a sucrose reward compared to their wt littermates and transgenic animals not over-expressing *Cdkn1c*, while perceiving the same reward as less palatable.

Chapter 6: *Cdkn1c* regulates social stability

6.1 Introduction

The functions of imprinted genes frequently converge on biological processes important in mammals, and evidence is growing to suggest this includes social behaviour (Isles et al., 2006; McNamara and Isles, 2014). As well as the effects of *Peg1, Peg3* and *Ube3a* on mother-offspring behavioural interactions and reproductive behaviour detailed in Chapter 1, imprinted genes have been shown to modulate adult social behaviours. Animals with a loss of neural expression of the imprinted gene *Grb10* were significantly more dominant towards unfamiliar mice in a tube test task and 'barber' their cage-mates more frequently than wt animals (Garfield et al., 2011).

Group housed male mice establish a linear, transitive, social hierarchy with a single dominant individual and a number of subordinates, and this hierarchy is stable across time (Ebbesen et al., 1992; Avitsur et al., 2007; Wang et al., 2011a). This hierarchical relationship is likely to minimise serious in-cage fighting (Hurst et al., 1993) and has positive benefits for individuals' health (Sapolsky, 2005). As a feature of this stability, an animal's rank in one measure of dominance can be correlated to its rank in another measure (Wang et al., 2011a). Dominance over other animals ensures better feeding (Cordero and Sandi, 2007; Wang et al., 2011a) and mating opportunities (Nelson et al., 2013) as well as access to other reinforcing stimuli (Vargas-Perez et al., 2009) and additional health benefits (Ebbesen et al., 1992; Moles et al., 2006; Sa-Rocha et al., 2006; Golden et al., 2011).

Dominancy can be defined, in a dyad, as one individual (subordinate) being submissive to another (dominant) (Drews, 1993). The classic task for determination of dominant and subordinate relationships between two animals is the tube test (Lindzey et al., 1961; Garfield et al., 2011; Noain et al., 2013), although this has come under criticism as a measure of dominance in recent years (Curley, 2011). An aspect of the definition of dominance described by Drews (Drews, 1993) is the priority of access to resources of one individual over

others. Competition for restricted water access is a representative scenario of the natural environment where resources are limited and dominancy over other individuals has clear benefit for survival. A variation of this task has been previously used to assess dominant-subordinate relationships in rat dyads and has shown to be a stable measure (Lucion and Vogel, 1994; Cordero and Sandi, 2007). Harnessing innate murine behaviours is a robust strategy for assessing dominancy. Mice use scent marks as a communicant of dominance to intruders (Hurst, 1993; Arakawa et al., 2007; Arakawa et al., 2008) and singly housed animals will increase or decrease scent marking behaviour to the scent of a dominant (or other singly house animal) or subordinate mouse, respectively (Arakawa et al., 2009). Therefore the animal's propensity to scent mark is an adaptive behaviour. In the presence of a cage mate and, in addition to being reflective of encounters in the natural environment, is provides an indicator of the dominant/subordinate relationship between the two individuals. Importantly this behaviour has previously shown, within cage groups, to correlate with other measures of dominance (Jemiolo et al., 1992; Drickamer, 2001; Wang et al., 2011a; Noain et al., 2013).

In this chapter, the consequence of increased *Cdkn1c* expression on dominance behaviours towards unfamiliar animals in a tube test task was examined. In order to probe this in more detail, tasks examining dominance were carried out which were representative of the normal social setting. Dominance behaviours within the home cage group, using three separate tasks were performed. A within-cage tube test, scent marking performance and duration of access to restricted water assessment was carried out in order to asses if *Cdkn1c* influenced rank within a social group or structure of the social hierarchy. In order to rule out any possible confounding factors, olfactory function was also assayed as indexed by latency to sniff a hidden cookie. Basal stress levels in these animals were assayed by examining serum levels of constituents of the HPA axis, corticosterone and adrenaline.

6.2 Methods:

6.2.1 Animals and indicators of fighting

Male mice were group housed from weaning at 3-4 weeks, with between two and five animals per cage. Each cage consisted of transgenic animals (*Cdkn1c*^{BACx1} or *Cdkn1c*^{BACLacZ}) and their wt littermates. All were housed in a 12:12 hours light:dark cycle with food and water provided *ad libitum* except under conditions described below. Details of general housing and husbandry are as described in Chapter 2.1. Animals were between 8-12 weeks at beginning of testing. Coat condition and general appearance was monitored regularly from weaning. Incidences of injury due to bullying/fighting were recorded where there were apparent, fresh wounds on the flanks or in the anogenital region. For the unfamiliar animal tube test task experiment n=71 animals were used in total, genotype Cdkn1c^{BACx1} (n=23) and their wt littermates (n=19), Cdkn1c^{BAClacZ} (n=14) and their wt littermates (n=15). For all remaining experiments n= 48 animals were used in total, Cdkn1c^{BACx1} (n=12) and their wt littermates (n=11), Cdkn1c^{BAClacZ} (n=16) and their wt littermates (n=9).

6.2.2 Tube test

The tube test was carried out as described in Garfield et al., (2011). Briefly, test apparatus consisted of a 30 cm smooth, transparent Perspex tube with a 3.5 cm diameter placed in a opaque Perspex box 13x28x45 cm in dimension to obscure view of the environment. Testing was carried out in dimmed light conditions. At the beginning of each trial two animals were introduced into the tube from both ends and released simultaneously. A trial was considered complete when one animal backed out and body and head were fully outside the tube. The animal that did not back out was considered the dominant animal of the trial. In every trial performed, one animal backed out before the other. Between trials the tube was wiped with a clean, dry, piece of tissue to remove any previous animals' odour and the tube was scented with soiled bedding from a cage of female C57BL/6 mice to further mask any previous animals' odour and to incentivise animals to enter. Apparatus was free from bedding at beginning of each trial.

6.2.2.1 Unfamiliar animal

All animals were naïve to the task prior to testing, as in Garfield et al., (2011). Experiment was carried out as described above; every trial consisted of one transgenic animal and one, weight matched, unfamiliar, wt animal. Each transgenic animal had three novel encounters and genotype of the winning animal was recorded for statistical analysis.

6.2.2.2 Within cage

For the duration of the experiment the home cage bedding remained unchanged. To exclude the effect of anxiety to a novel environment, animals were trained individually to pass through the tube for two day prior to testing. On test days all animals faced each of its cage mates in a 'round robin' design and this was carried out for four consecutive days. For each day an animal was given a rank depending on the number of encounters won, the animal that did not back down in any of its trials was considered to be the most dominant animal in the cage and was given a rank of 1 and so on to the animal that backed out first in all of its trials which was assigned the lowest rank. After four days each animal had an average rank score.

6.2.2.3 Bedding change

This experiment followed the same protocol as the within cage tube test. Animals' rank was assessed in four sessions; on day one, for refamiliarisation with the task and exclude effects of novelty on results, (E1.1), day two in the morning (E1.2) and in the afternoon (E1.3) and day three in the afternoon (E1.4). Between E1.2 and E1.3 the home cage was cleaned, animals were moved to a new cage and all bedding was replaced to remove any odours identifying the previously dominant animal in the group. E1.3 and E1.4 were carried out 1 hour and 24 hours, respectively, after cage change. For statistical analysis, whether an animals' rank differed over a environment change (E1.2 to E1.3) compared to when there was no change in the environment (E1.3 to E1.4) was recorded as '0' for no change in rank and '1' for rank changed, regards of which direction the changed occurred in.

6.2.3 Scent marking

For this experiment a black Perspex box 30x30x30 cm was bisected by a wire mesh 20x29.5 cm (grid size 0.6x0.6 cm) topped with transparent perspex 14x29 cm. Both sides of the floor was lined with absorbent paper (3MM Whatman, Fisher Scientific). See Appendix B for schematic of experimental set up. Each encounter consisted of one animal and a cage-mate placed on either side of the wire mesh, through which was they could receive visual, auditory and olfactory information but could not physically interact. The experiment was carried out under dim lighting conditions and each encounter lasted one hour. Each animal met all of its cage mates in such an encounter, with each animal having not more than one encounter per day. Scent marks made on absorbent filter paper were visualised under ultraviolet light and outlined by pencil. Analysis was modified from Arakawa et al., 2007. Briefly, a grid of 1 x 1 cm squares was overlaid and the number of squares containing scent marks (maximum 420 squares) was recorded for each animal for each encounter. Marks greater than 4 squares in size were excluded. The dominant animal in the encounter was considered to be the animal that scent marked more than its opponent. Each animal was assigned a rank depending on the number of encounters won.

6.2.4 Competition for water access

For this task animals had restricted water access, provided for two hours per day, immediately after testing. Animals were trained individually to locate and consume freely available water, provided though a metal drinking spout in a 600 s trial in a Phenotyper arena (Noldus Information Technology). After three training sessions, animals were placed in the Phenotyper arena in their full cage group and drinking spout was introduced. The trial was digitally recorded and duration each animal spent drinking was scored manually offline. Each animal was given a rank depending on the duration of water access obtained during the first 120 s of and the full 600 s trial. Each animal was assigned a rank depending on the duration of water access obtained in the 120 s and the full 600 s.

6.2.5 Olfactory function

A cookie was hidden under sawdust in an open field arena (30 x 30 cm and illuminated evenly with a 60 W bulb) the floor of which was completely covered in clean sawdust. Animals were placed in the opposite quadrant to the cookie and the quadrant was changed for each successive animal. Activity was tracked using a camera connected to a computer with ETHOVISION software (Noldus, Nottingham, UK) and latency to sniff the cookie (defined to be when the middle of the animal was within 1 cm of the odour) was recorded.

6.2.6 ELISA

Serum was collected as described in Chapter 2.1.4. Serum corticosterone (DRG Instruments GmbH, Marburg, Germany) and epinephrine (UscnLife Science Inc., Wuhan, China) ELISAs were carried out according to the manufacturers instructions. 10 µl of serum per sample was loaded in duplicate for corticosterone ELISA. 50 µl of serum per sample was loaded in duplicate for epinephrine ELISA.

6.2.7 Statistical analysis

All statistical analysis was carried out using SPSS 20.0 (SPSS, USA). Dominance behaviours towards an unfamiliar animal in the tube test were assessed by carrying out a non-parametric chi-squared test, assuming all groups were equal. For analysis of genotype on rank within a cage group, each animal's rank within the cage was transformed to a number between 0 (least dominant animal in the group) and 1 (most dominant animal in the group), this was performed for each group for each task to allow for differences in cage group size. A non-parametric Mann-Whitney U test was carried out with GENOTYPE as the grouping variable. Correlation between ranks is different measures of dominancy was determined using a non-parametric Spearman's rank-order correlation and statistical difference between correlation coefficients was assessed using Fisher r-to-z transformation. For analysis of effect of genotype on likelihood to be involved in severe fighting, a chi-square cross-tabs test was carried out with GENOTYPE as rows and BITE PRESENCE as column. Rank stability across a change in environment, rated as '0' for no changed and '1' for rank change, was assessed using a conditional logistic regression, regressing on CAGE, to take into account

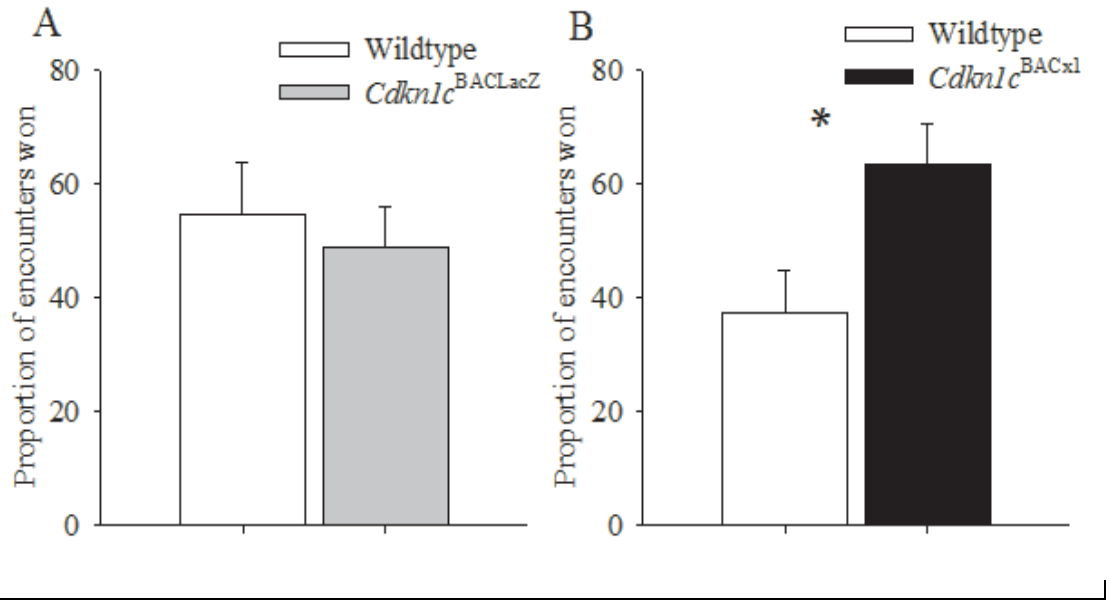
cage group sizes. A series of ANOVAs was used to analyse olfactory function and serum hormone levels.

6.3 Results

6.3.1 Cdkn1c^{BACx1} animals were significantly more dominant towards unfamiliar animals in a tube test than their wt littermates

The average weight on test day by genotype was Cdkn1c^{BACx1} (32.6 g SEM 0.4 g), Cdkn1c^{BACx1} wt (33.5 g SEM 0.6 g), Cdkn1c^{BACLacZ} (27.9 g SEM 0.4 g), Cdkn1c^{BACLacZ} wt (28 g SEM 0.6 g). There was no significant difference in the proportion of encounters won by *Cdkn1c*^{BAClacZ} animals (46.7%) compared to wt animals (53.3%, χ^2=0.2, p=0.655) (figure 6.1B). However, animals with a double dose of *Cdkn1c* won significantly more encounters than wt animals (63.1 vs 36.9%, χ^2=4.45, p=0.035) (Figure 6.1A). Simply increasing the dosage of *Cdkn1c* two fold caused increased dominance towards unfamiliar animals, implicating a role for *Cdkn1c* in positively regulating social dominance.

Figure 6.1: *Cdkn1c*^{BACx1} males were more dominant towards unfamiliar males in a tube test compared to wt. A) There was no difference in proportion of encounters won between *Cdkn1c*^{BACLacZ} and wt animals. B) *Cdkn1c*^{BACx1} animals won significantly more encounters against unfamiliar animals in the tubes test than wt. Data shown are means +/- SEM



6.3.2 The stable in-cage social hierarchy is disrupted in the presence of a Cdkn1c^{BACx1} male.

There was no effect of genotype, either with *Cdkn1c*^{BACx1} (Tube test: U=57, Z=-0.557 p=0.608; Scent marking: U=89, Z=-0.05 p=0.981; water access: U=67.5, Z=-0.873 p=0.403) or *Cdkn1c*^{BACLacZ} (Tube test: U=64, Z=-0.455 p=0.677; Scent marking: U=51.5, Z=-1.187 p=0.251; water access: U=63, Z=-0.523 p=0.637) animals, on the average group rank in any measure of dominancy examined (Figure 6.2). In groups of *Cdkn1c*^{BACLacZ} animals and their wt cage mates, an individual's rank in one measure of dominance was significantly correlated with its rank in another measure, specifically, tube test vs. water access in the first 120s rank (Spearman's ρ correlation= 0.493, p=0.012) and scent marking vs. water access 600 s rank (Spearman's ρ correlation = 0.597, p=0.002) (Figure 6.3A, C). In contrast, groups of *Cdkn1c*^{BACx1} and wt animals did not have a stable social hierarchy. A clear transitive hierarchy was apparent in each task. However, in the presence of a *Cdkn1c*^{BACx1} male an individual's rank in one measure of dominance was not correlated with its rank in another (tube test vs. water access in the first 120s rank, Spearman's ρ correlation= -0.034, p=0.878 and scent marking vs. water access 600 s rank, Spearman's ρ correlation = -0.134, p=0.513) (Figure 6.3B, D). Fisher r-to-z transformations showed there was a significant difference in the correlation coefficients between the groups (Tube test vs water access 120 s, z=2.05, p=0.04; Scent marking vs water access 600 s, z=2.54, p=0.01). These findings suggested that the presence of a *Cdkn1c*^{BACx1}animal in a group destabilised the social structure, resulting in more frequent rank changes.

Figure 6.2: *Cdkn1c* over expression does not generally affect dominance behaviours within the social group (i.e. within cage). The was no effect of genotype on the average group rank in the within cage tube test (A, D), the scent marking task (B, E) and the water access task (C, F). Data shown are means +/- SEM

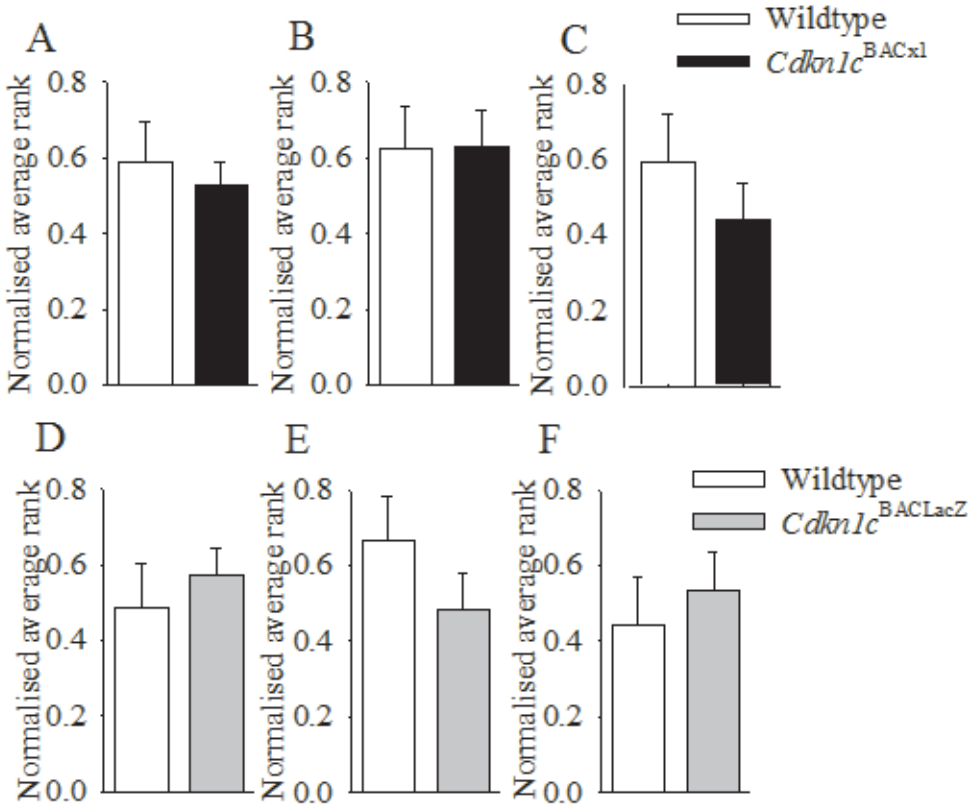
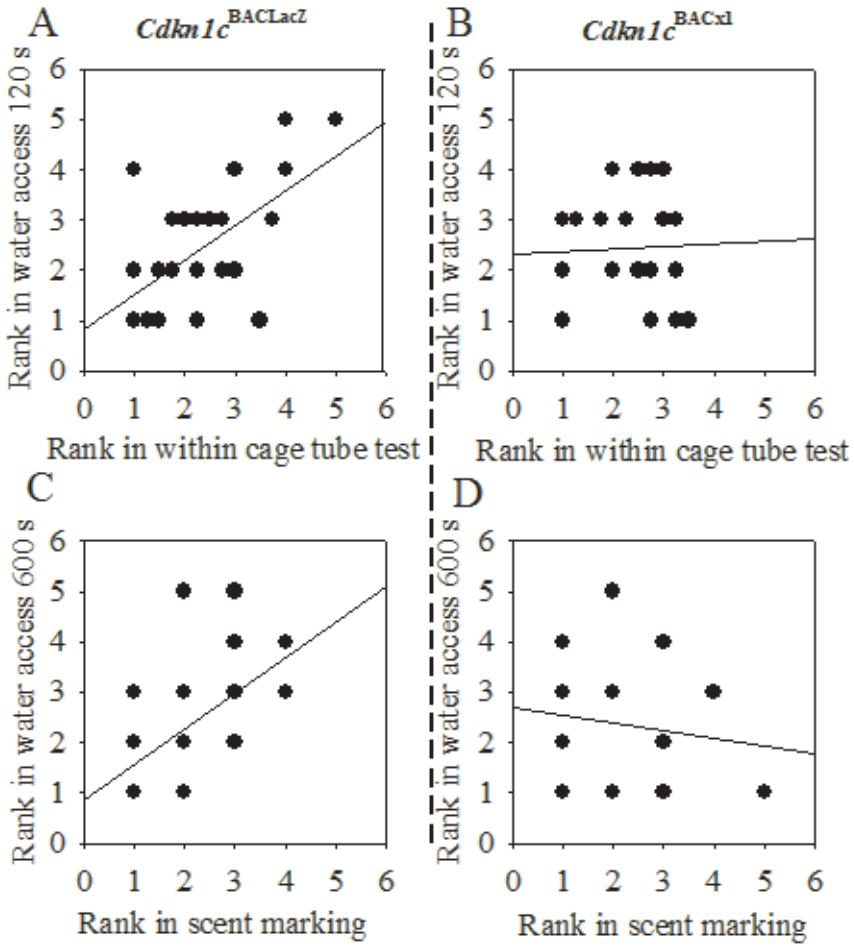


Figure 6.3: Presence of a *Cdkn1c*^{BACx1} male destabilises the social hierarchy. In cages of *Cdkn1c*^{BACLacZ} and wt animals rank in the within cage tube test is correlated with rank in the water access task in the first 120 s (A). Additionally, rank in the scent marking task is correlated with rank in the water access task in 600 s (C). This was not the case in groups containing *Cdkn1c*^{BACx1} males where there was no correlation between rank in the within cage tube test is correlated with rank in the water access task in the first 120 s (B) nor rank in the scent marking task is correlated with rank in the water access task in 600 s (D).



6.3.3 Cdkn1c^{BACx1} animals were more likely to change position in hierarchy than their wt littermates after removal of odour cues

In a stable social hierarchy, variability in rank is not expected to significantly differ when odour cues are removed and the social hierarchy must be re-established. This was the case for cages containing *Cdkn1c*^{BACLacZ} animals and their wt littermates (Conditional logistic regression, Wald statistic=0.665, p=0.415), compared to when the environment remained stable (Conditional logistic regression, Wald statistic=0.010, p=0.922) (Figure 6.4A). *Cdkn1c*^{BACx1} animals had significantly more variable rank compared to their wt cage mates when odour cues were removed (Conditional logistic regression, Wald statistic=3.925, p=0.048) (Figure 6.4B). This was not the case when the environment was unchanged, as there was no difference in the rank variability between genotypes (Conditional logistic regression, Wald statistic=0.021, p=0.886). This implies that when the cues indicating the dominant animal in the group were removed, *Cdkn1c*^{BACx1} animals were more likely to challenge for dominance, resulting in a more variable rank.

Importantly, the effects on social stability were unrelated to olfactory function as there was no difference between *Cdkn1c*^{BACx1} individuals and either their wt cage mates or *Cdkn1c*^{BACLacZ} animals, in ability to detect presence of a cookie odour (*Cdkn1c*^{BACx1} main effect of GENOTYPE $F_{1,27}$= 0.719, p=0.404, *Cdkn1c*^{BACLacZ} main effect of GENOTYPE $F_{1,29}$= 1.474, p=0.235) (Figure 6.5A, B)

Figure 6.4: When odour cues indicating dominance were removed, *Cdkn1c*^{BACx1} males change their rank more frequently than wt. In groups of *Cdkn1c*^{BACLacZ} and wt animals, rank fluctuation did not differ when olfactory cues indicating the dominant animal were removed (A, left) nor when the environment remained stable (A, right). When odour cues were removed *Cdkn1c*^{BACx1} males' rank fluctuated significantly more than wt animals (B, left). This was not the case when the environment remained stable (B, right). Data shown are means +/- SEM

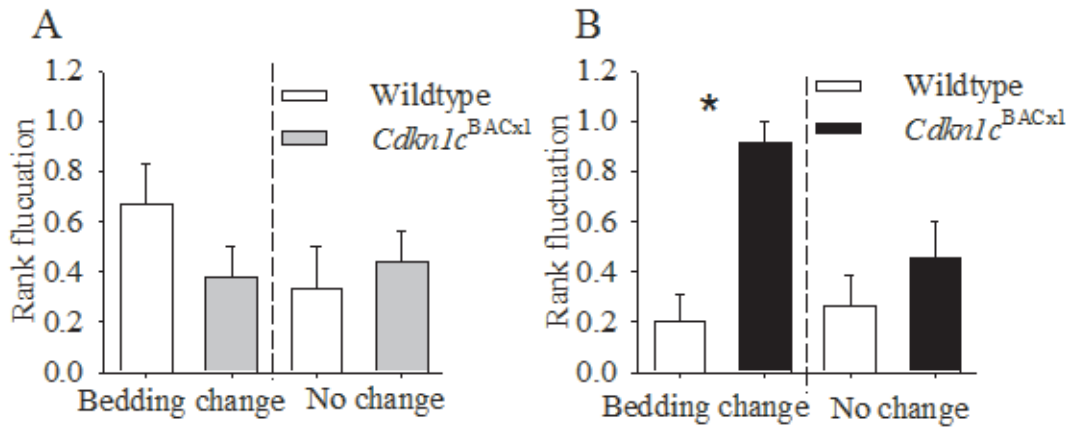
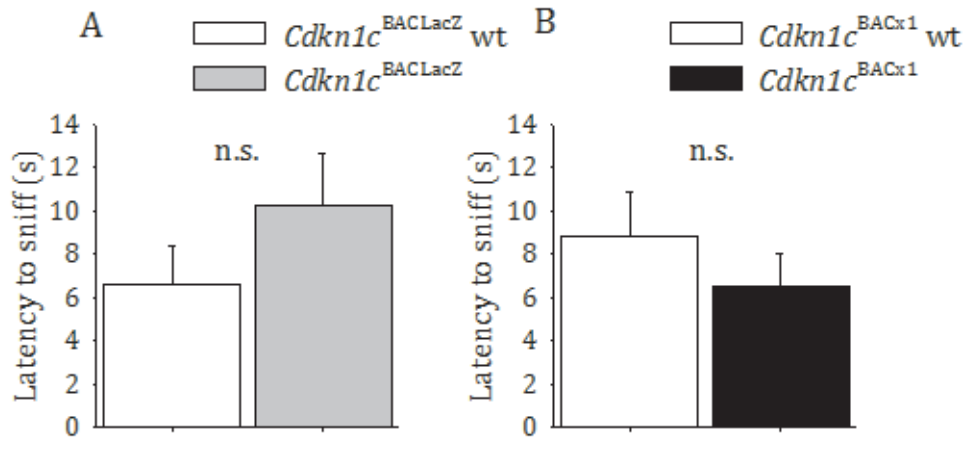


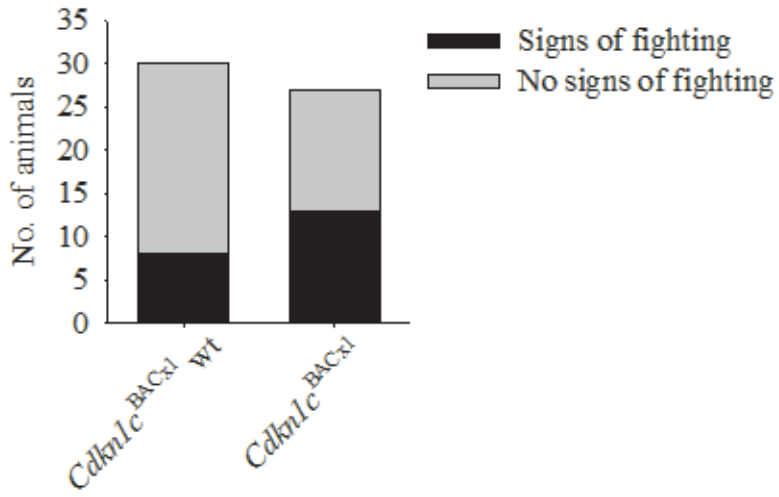
Figure 6.5: There is no difference between groups in the ability to detect an odour. All animals could detect the presence of an odour (hidden cookie) equally well. Data shown are means +/- SEM



6.3.4 A consequence of an unstable social environment is potentially reduced fitness.

There were significantly more signs of serve in-cage fighting (fresh cuts along flanks or in ano-genital region observed on at least one occasion) in cages containing animals over expressing *Cdkn1c* and their wt cage mates (36.8% of animals, n=50) compared to cages of *Cdkn1c*^{BAClacZ} animals and their wt cage mates (no observed occurrences, n=56) and cages containing only wt littermates of *Cdkn1c*^{BACx1} animals (no observed occurrences, n=7). Signs of serve fighting were not different by genotype (pearson χ^2 = 2.818, p=0.093) (Figure 6.6), indicating that presence of a *Cdkn1c*^{BACx1} in a group had negative effects on fitness for both the transgenic and wt animals in that group.

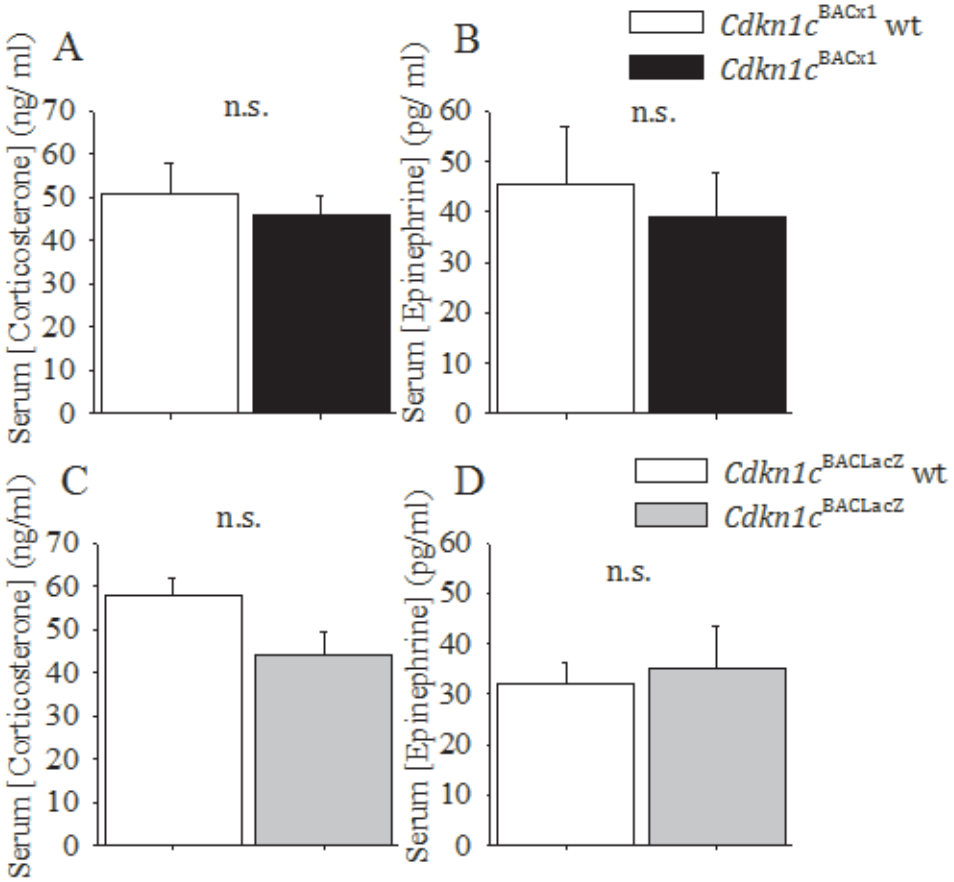
Figure 6.6: There is no effect of genotype on likelihood to be receive severe wounds



6.3.5 Serum stress biomarker levels

There was no difference between *Cdkn1c*^{BACx1} and animals and their littermates, nor between *Cdkn1c*^{BACLacZ} and their littermates, in resting levels of serum metabolic stress indicators, corticosterone (*Cdkn1c*^{BACx1} $F_{1,24}$= 0.437, p>0.05, *Cdkn1c*^{BACLacZ} $F_{1,25}$=3.041, p>0.05) and epinephrine (*Cdkn1c*^{BACx1} $F_{1,22}$= 0.187, p>0.05, *Cdkn1c*^{BACLacZ} $F_{1,24}$=0.056, p>0.05) (Figure 6.7).

Figure 6.7: There is no effect of genotype on basal serum corticosterone (A,C) or epinephrine (B,D) levels. Data shown are means +/- SEM



6.4 Discussion

This chapter set out to examine the social behaviour of *Cdkn1c*^{BACX1} mice. The experiments demonstrated that two fold over expression of *Cdkn1c* was sufficient to increase dominancy of males towards unfamiliar animals in a tube test. In an identical task, ablation of the paternally expressed *Grb10* allele resulted in a similar phenotype (Garfield et al., 2011). These results imply that these oppositely imprinted genes may exert antagonistic actions on social dominance behaviours.

These experiments assessed key aspects of social behaviour using a number of different measures within the normal social environment (home-cage group) in order to establish the position of each individual within the social hierarchy. Intriguingly, these tests did not identify *Cdkn1c*^{BACx1} animals as being generally ranked higher within the social group hierarchy as might be expected from a generally more dominant animal. Instead, the presence of a *Cdkn1c*^{BACx1} animal in a cage group resulted in an unstable social hierarchy. This unstable ranking suggested that, while *Cdkn1c*^{BACx1} animals were not more socially dominant *per se*, they were more likely to challenge for dominancy, a phenotype not observed in the control groupings with, *Cdkn1c*^{BAClacZ} mice. Moreover, the variable rank in the cage hierarchy across time and the disruptive presence of *Cdkn1c*^{BACx1} had a consequence for fitness of the whole group.

6.4.1 Cdkn1c regulates behaviour towards unfamiliar animals in a tube test task

In the previous study on ablation of the paternally expressed *Grb10* allele, increased likelihood of winning in a tube-test encounter with unfamiliar animals was interpreted as being indicative of altered social dominance behaviour (Garfield et al., 2011). *Cdkn1c*^{BACx1} mice similarly displayed an increased likelihood of winning in a tube-test encounter with unfamiliar animals, suggesting an antagonistic action of these two genes on this behaviour. However, use of the tube test as a measure of dominance has come under some criticism (Curley 2011). Garfield and colleague's conclusion of increased dominance was based on performance in a tube test task and the observation that Grb10^{PATKO} animals 'barbered' their cage mates more frequently. Whisker

'barbering' has been implicated as a sign of dominance within cage groups (Sarna et al., 2000; Garfield et al., 2011; Haig and Úbeda, 2011). There was no observed occurrence of barbering between *Cdkn1c*^{BACx1} animals and their wt cage mates. This could suggest a more subtle role for *Cdkn1c* in regulating social behaviour.

6.4.2 Presence of an animal over expressing Cdkn1c has negative consequences on the stability of the social hierarchy

In order to examine the social group structure it was necessary to establish the rank of each individual within the social group. Each animal's performance was assessed in a test of scent marking in the presence of a cage mate, access to a limited water access over other members of the social group and the tube test with familiar males in order to determine the dominant and subordinate relationships between animals within their social group. This revealed the expected linear hierarchy (Wang et al., 2011a) in the majority of social groups for both *Cdkn1c*^{BACx1} and their wt littermates and *Cdkn1c*^{BACLacZ} and their wt littermates. While an animal's rank was stable across time and measures in the case of *Cdkn1c*^{BACLacZ} and their wt littermates, in the presence of an animal over expressing *Cdkn1c* there was a loss in rank stability over time.

This finding is unlikely to be a product of individual animals' aptitude for a task. If that were the case, there would be an ill defined hierarchy with a single dominant/subordinate animal being apparent only as an artefact of task aptitude. This was not the case. In the within cage tube test, a clear, linear, transitive, hierarchy was apparent for an average of 3.4 days out of 4 in groups of *Cdkn1c*^{BACLacZ} animals and their cage-mates and an average of 3.3 days out of 4 in groups of *Cdkn1c*^{BACx1} animals and their cage mates. Similarly, a clear hierarchy was apparent in 100% of cages containing *Cdkn1c*^{BACLacZ} animals and in 85.7% of cages containing *Cdkn1c*^{BACx1} animals in the competition for water access task. A clear hierarchy was also apparent in 71.4% cage of cages containing *Cdkn1c*^{BACLacZ} animals and in 57.1% of cages containing *Cdkn1c*^{BACx1} animals in the scent marking task. This shows that the absence of a relationship between measures of dominance in groups containing *Cdkn1c*^{BACx1} males was

not due to a defect in ability to establish a hierarchy but is a consequence of more frequent rank fluctuations.

The tasks used were designed to mimic conflict in the natural environment, territory marking and competition for limited water access. Initially the tube test task was carried out for ten days, as previously described (Wang et al., 2011a). However, there was not a significant difference in the average rank after testing for four days compared to ten days. Therefore, all testing was carried out for four consecutive days after two days training to reduce time without a cage cleaning. At least one cage bedding change occurred between each task.

Though aggressive behaviours have been demonstrated to be increased after water deprivation (Tucci et al., 2006), this did not translate to increased dominancy in the tube test (Tucci et al., 2006). Additionally, it has been shown that restricted food intake does not affect the stability of the dominance hierarchy, when animals were housed in low numbers (Ebberson et al., 1992), as was the case in these experiments. Activity intensity during the competition for water access changed across the duration of trial, appearing more aggressive in the initial 120 s, and as such, we chose to look at two time bins.

As would be expected in a stable social hierarchy, in cages of *Cdkn1c*$^{\text{BACLacZ}}$ and wt animals, rank was correlated between the within cage tube test task and the water access task for the initial 120 s. Additionally, rank in the scent marking task was correlated with rank in the water access task across the full 600 s trial. This was not a reciprocal relationship as there was not a significant interaction between either an animals' rank in the scent marking task and its rank in the water access task in the first 120 s of the trial (Spearman's ρ correlation = 0.3, $p>0.05$, data not shown) nor animals' rank in the within cage tube test and its' rank in the water access task across the full 600 s (Spearman's ρ correlation = 0.02, $p>0.05$, data not shown). This likely reflects the different aspects of dominancy each task entails, with a stronger relationship between more confrontational behaviours (tube test, water access task 120 s) and behaviours

involving asserting dominance over a longer period (scent marking, water access 600 s). Nonetheless, these finding were temporally separated and thus highlight the stability of the established hierarchy.

6.4.3 Cdkn1c^{BACx1} males change rank more frequently with negative consequences for fitness for social group members

Dominancy relationships in groups, while generally stable, can change under pressurising circumstances (Cohn et al., 2012). The observed loss of rank stability may be as a consequence of a greater propensity of *Cdkn1c*$^{\text{BACx1}}$ animals to challenge for dominance. The hierarchy must be re-established when odour cues indicating the dominant animal are removed (Gray and Hurst, 1995; Van Loo et al., 2000). In a stable social structure, removal of odour cues should not perturb an animal's rank any greater than in an unchanging environment. Using the tube test, this was shown to be the case in groups of *Cdkn1c*$^{\text{BACLacZ}}$ animals and their wt cage mates. However, *Cdkn1c*$^{\text{BACx1}}$ males changed rank after removal of odour cues more frequently than their wt cage mates, implying that, when the social hierarchy must be re-established, these animals were more likely to challenge for dominance. This finding potentially explains why, within groups of *Cdkn1c*$^{\text{BACx1}}$ animals and their wt cage mates, there was no significant relationship between an animal's rank in different measures of dominance. In an established home cage group, this increased propensity to challenge for dominance will not necessarily result in a higher rank in the hierarchy but is likely to lead to increased disruption, something evidenced by the increased incidence of in-cage fighting. This idea may also shed light on the findings in a tube test with an unfamiliar animal, where the increased propensity to challenge for dominancy may manifest as a decreased likelihood to back down to a novel conspecific, resulting in *Cdkn1c*$^{\text{BACx1}}$ appearing to display more dominant behaviours compared to unfamiliar wt animals.

Presence of an animal over expressing *Cdkn1c* had consequence for fitness for the whole group, with increased signs of fighting in cages containing these animals. The wounds were unlikely to be self-inflicted or due to over-grooming

given their location as characteristic targets (flanks, anogenital region) of inter-individual aggression. However, observations were of relatively severe encounters and are limited in that they do not record the number of inter-individual encounters that did not result in flesh wounds.

6.4.3 Conclusions

Cdkn1c, a maternally expressed gene, appears to function antagonistically to *Grb10*, a neurally paternally expressed gene, in regulation of dominance behaviour towards an unfamiliar animal in a tube test task. However, *Cdkn1c* does not appear to regulate dominance, *per se,* as these animals did not have a higher rank within the social group than wt animals. *Cdkn1c*^{BACx1} animals appeared to disrupt the stability of the social hierarchy by more frequently challenging for dominance. This was not confounded by olfactory function or basal stress hormone levels. A stable social group has benefits for individual animals, as well as the group as a whole. A failure to adhere to social 'norms' by animals over expressing *Cdkn1c* provides a fascinating insight to the function of this imprinted gene, which will be discussed later.

Chapter 7: The in utero environment and Cdkn1c

7.1 Introduction

Previous studies examining the consequences of a prenatal and lactational low protein diet reported an increase in *Cdkn1c* expression in the adult brain alongside relative hypomethylation of *Cdkn1c*-DMR and alterations in the dopaminergic system (Vucetic et al., 2010b). This chapter focused on further exploring the *in utero* environment in relation to expression of *Cdkn1c* and other imprinted genes with a known role in neurodevelopment. Given the profound phenotypes observed after approximately 2-fold increased expression induced in the transgenic model (this work, chapters 3-6), altered expression of *Cdkn1c* in response to an environmental challenge may have profound consequences for adult behaviours. In addition to dietary alterations, *Cdkn1c* has been shown to be increased at P6 in the ventral midbrain of pups exposed to high maternal licking and grooming (Jensen Peña et al., 2014). Both of these studies observed accompanying changes in the dopaminergic system and in dopamine related behaviours. While both studies examined expression, and in one case methylation (Vucetic et al., 2010b), neither determined the allelic origin of increased expression. This is especially relevant as it has been shown that methylation of *Cdkn1c* DMR does not necessarily correlate with allelic expression (Wood et al., 2010). Elevated *Cdkn1c* may consequently reflect either an increase in the number of cells expressing *Cdkn1c*, further activation of the maternal allele or a failure to silence, or maintain silencing, of the paternal allele.

Correlating with the observed sensitivity of *Cdkn1c* expression to gestational environment were changes in the dopamine system. Indeed, the sensitivity of the dopaminergic system to the gestational environment is well established. Adult rats exposed to prenatal stress were more sensitive as adults to low doses amphetamine in a self administration paradigm (Hausknecht et al., 2013). This was accompanied by a reduced number of spontaneously firing DA VTA neurons after prenatal stress (Hausknecht et al., 2013). This condition has also been found to be linked to an increase in Drd2 receptors in a region specific manner

(Berger et al., 2002). *Dat* has also been found to be sensitive to the prenatal environment. Dat was found to be increased after prenatal exposure to high doses of ethanol in the adult mouse cortex and striatum (Kim et al., 2013b). *In utero* and pre-weaning exposure to a low protein diet, in addition to an elevation in *Cdkn1c*, has been shown to increase *Th* and *Dat* expression in the adult male VTA and nucleus accumbens (Vucetic et al., 2010b). This was accompanied by an increase in Th positive cells in the VTA, increased dopamine turnover in the PFC and altered dopamine related behaviours (Vucetic et al., 2010b). This prenatal condition was also associated with increased haloperidol binding in the striatum, specifically in females (Palmer et al., 2008), indicating an increased in the number of dopamine receptors. Similarly, obesity at conception and/or a high fat diet during gestation and lactation caused an alteration in the adult male offspring in neural levels of dopamine related genes (*Th, Dat, Drd1, Drd2, Darrp-32*), in a region specific manner (Vucetic et al., 2010a; Grissom et al., 2013). Dat, as well as Drd1, Drd2 and Drd3, were also found to be altered after prenatal amphetamine exposure in a regional and age dependant manner (Flores et al., 2011). Finally, prenatal under nutrition, by restriction of dam food intake to 50% for the final gestational week, additionally resulted in region and age specific changes in Drd1 and Drd2 levels (Manuel-Apolinar et al., 2014). This highlights the complexity of investigating prenatal stressors, due to the possibility for any potential phenotype to interact with the postnatal environment. Of particular relevance is the observation that dietary manipulations during lactation alone can produce a phenotype in a progressive ratio task (Reyes-Castro et al., 2012a).

The previously reported results linking maternal diet to elevated neural *Cdkn1c* (Vucetic et al., 2010b) were potentially confounded by the effects of the postnatal environment both with respect to the continuation of the low protein diet through lactation and a possible maternal behavioural phenotype in this environment. Similar consequences for *Cdkn1c* expression have been reported after differing maternal care in the first postnatal week (Jensen Peña et al., 2014). In addition, this work (Chapter 6) and previous indirect work on elevated *Cdkn1c* (Jensen Peña et al., 2014) have, through correlation but not

necessarily causation in the case of the latter study, implicated social behaviours as being regulated by dosage of this gene. Therefore, this work examined the consequences of exposure to an altered prenatal diet on *Cdkn1c* and other imprinted gene expression at a late embryonic time point prior to introduction of postnatal confounding factors such as maternal care and/or interaction with peers.

This chapter focused on the consequences of a suboptimal maternal diet during pregnancy on neural expression of *Cdkn1c* in the offspring at E18.5. The work in this thesis (Chapters 3-6) examining the effects of specifically over-expressing *Cdkn1c*, suggests that the previously identified correlative changes in *Cdkn1c* and the dopamine system in response to maternal diet (Vucetic et al., 2010b) may be causally linked. In addition to *Cdkn1c,* a range of imprinted genes were assayed to determine the sensitivity/resilience of expression levels of these genes in the developing brain to gestational adversity. The imprinted genes examined were selected to represent several classes of imprinted loci, as well as being those shown to be sensitive to the prenatal environment (McNamara and Isles, 2014) and/or expressed in the brain (Wilkinson et al., 2007; Tunster et al., 2013). A late prenatal time point was chosen to combine study of a relatively mature nervous system and to avoid any potential interaction of phenotypes with the postnatal environment. As shown in this thesis in figure 3.1A, *Cdkn1c* expression declines in the brain from E13.5. Sampling at E18.5 should allow sufficient exposure to the prenatal insult to detect the potential consequences on *Cdkn1c* expression. The maternal diet manipulations were chosen as they had previously been linked to alterations in dopamine related or imprinted genes expression (Gong et al., 2010; Vucetic et al., 2010a; Vucetic et al., 2010b; Grissom et al., 2013; Sferruzzi-Perri et al., 2013). The dopaminergic system state at this time point was also examined, at the mRNA, protein and neurotransmitter level, in order to determine the resultant effects of the altered maternal diet before interaction of any phenotype with the postnatal environment.

7.2 Methods

7.2.1 Animals and diet

Animals were housed as described in Chapter 2.1.2. Wt C57BL/6 female mice, aged between 7 and 10 weeks, were used. These were paired with a *Cdkn1c*-RFLP male maintained on the C57BL/6 background. These mice carry a spretus copy of the distal chromosome 7 region as a result of back crossing the out-bred strain *Mus spretus* onto a C57BL/6 background for >10 generations, selecting for an *AvaI* RFLP polymorphism in *Cdkn1c*. Once a vaginal plug was recorded, females were singly housed and fed either a 8.5% protein (dietary energy from; protein 8.1%, fat 21.8%, carbohydrate 70.1%) (Test Diet, St. Louis, MO), 45% fat (dietary energy from; protein 18.1%, fat 46.1%, carbohydrate 35.8%) (Test Diet, St. Louis, MO) or a control basal diet (diet energy from; protein 18.3%, fat 22.1%, carbohydrate 59.6%) (Test Diet, St. Louis, MO) from E0.5. For full dietary composition see Appendix C.

7.2.2 Dissections

Pregnant dams were sacrificed by cervical dislocation at E18.5 after the observation of a vaginal plug. The abdominal cavity was exposed and the uterine horn was removed into ice cold sterile PBS. Pups and placenta were weighed and placenta was snap frozen for future analysis. Pups were decapitated and kidneys were removed and snap frozen. Embryo brains were removed from the skull and cerebellum and hindbrain were separated from the fore- and mid-brain. Both were bisected along the midline into left and right hemispheres and snap frozen. All tissue was stored at -80°C until further processing. Four litters per condition were generated and tissue analysed was balanced for litter and for sex.

7.2.3 qPCR

RNA was extracted, DNase I treated and converted to cDNA as described in Chapter 2.2. qPCR was carried out on Corbett rotor gene 6000 (now supplied and maintained by Qiagen) using the mastermix template outlined in Chapter 2.2.4. Genes of interest fell into two categories, imprinted genes and genes of the dopaminergic system. Imprinted genes assayed were *Cdkn1c, Grb10*, *Igf2*, *Dlk1*,

Nnat, *Peg3* and *Snord116*. Dopaminergic genes assayed were *Drd1, Th and DAT.* Each reaction was carried out in triplicate for a given sample. The number of samples in each group is indicated in the text. The ΔCt was obtained by normalising to the geometric mean Ct value of *βactin* and *Hprt* for that sample. The ΔΔCt was calculated by taking the ΔCt value for a given sample from the average basal diet ΔCt. Statistical analysis was carried out on these values. Fold change from basal diet was calculated using the formula $2^{-\Delta\Delta Ct}$.

7.2.4 High resolution melt analysis (HRM)

Parental allelic contribution to the *Cdkn1c* PCR product was carried out by HRM analysis of the *Cdkn1c* PCR product and *βactin* PCR product, as an unchanged control. PCR was carried out on Corbett rotor gene 6000 (now supplied and maintained by Qiagen) using the mastermix template outlined in Chapter 2.2.5. No data was acquired during the cycling steps. After 40 cycles, the temperature was stepped in 0.05°C increments from 80°C to 99°C, held for 2s at each step and fluorescence was recorded. The peak melt temperature for each PCR product was recorded and carried out in triplicate for each sample and was repeated on three separate occasions.

7.2.4 Western blot

Protein was extracted and quantified as described in Chapter 2.3. Protein concentrations were adjusted to 5 mg/ml and 75 µg per well was loaded. Protein separation by gel electrophoresis, transfer and Western blotting was carried out as described in Chapter 2.3. Primary antibodies were used to detect proteins of interest, specifically Th and βactin, at concentrations detailed in chapter 2.3.3. Relative protein abundance was calculated compared to βactin loading control using ImageJ (NIH, USA). Each western was repeated on two separate occasions, result was considered genuine only if there was agreement between replicates.

7.2.5 ELISA

Tissue levels of dopamine in the fore- and midbrain were assessed using a commercial ELISA kit (Stratech Scientific, UK). Tissue was homogenised in

homogenisation buffer (10 mM HCl 1mM EDTA 4mM sodium metabisulfite) and ELISA was carried out according to manufacturer's instruction. Each sample was carried out in duplicate and was repeated on two separate occasions. Data shown is from one representative example.

7.2.6 Statistical analysis

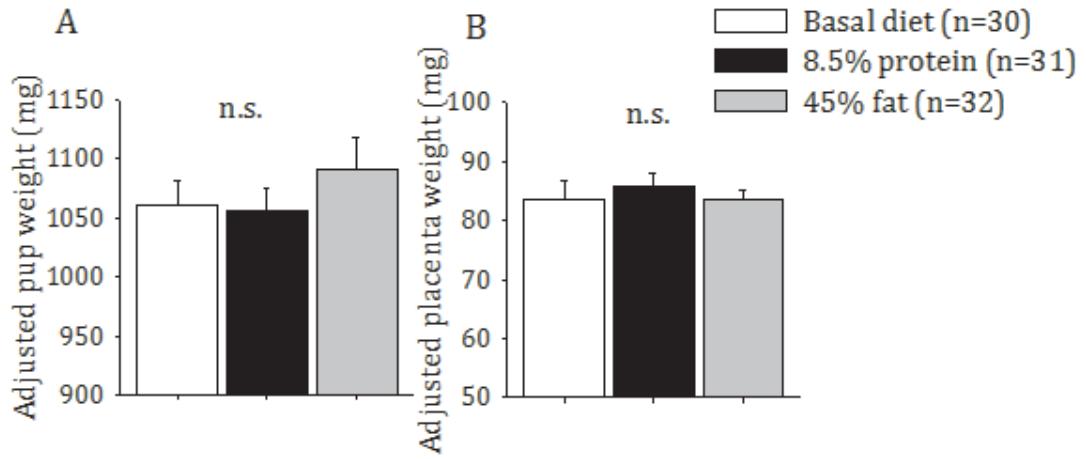
Pup and placenta weight were adjusted using the following formula to account for differences in litter size: ((average weight for diet)/(average weight for litter))*individual weight. An independent samples t-test was carried out between basal diet and each test condition individually with DIET as grouping variable. For qPCR and ELISA data Shapiro-Wilk test for normality was carried out on all ΔCt values. Values that were >3 SD from the group were excluded from analysis as outliers. Where data was normally distributed independent sample t-tests were carried out with DIET as grouping variable. Where the data was not normally distributed non-parametric Mann-Whitney U-tests were carried out. To correct for multiple comparisons in imprinted gene expression a Bonferroni corrected α was used for each test condition, namely α/7=0.0071. For protein abundance testing an independent samples t-test was carried out with DIET as grouping variable.

7.3 Results

7.3.1 Pup and placenta weights

There was no affect of maternal diet on litter size (basal: mean 7.5 SEM 0.65; low protein: mean 7.75 SEM 0.49; high fat: mean 8.0 SEM 0.91). There was no effect of maternal low protein (pup: $t(59)=-0.2$, $p=0.842$; placenta: $t(59)=-0.642$, $p=0.523$) or high fat (pup: $t(60)=-0.832$, $p=0.41$; placenta: $t(60)=-0.115$, $p=0.909$) diet on the adjusted placental or adjusted pup weight (figure 7.1 A, B).

Figure 7.1: There was no effect of maternal low protein diet or high fat diet on the adjusted (A) pup weight or (B) placental weight at E18.5. Data shown is mean ± SEM. Not significant (n.s.)



7.3.2 Imprinted gene expression after maternal low protein or high fat diet

In the brain, most of the imprinted genes surveyed were unaffected by gestational adversity, as indexed by no significant changes from basal diet condition. However, after maternal low protein diet there was a significant increase in expression of *Cdkn1c* in the fore- and midbrain of embryos ($t(22)= -3.236$, $p=0.004$) (Figure 7.2A). This was observed in both sexes. This appeared to be organ specific as there was no difference in *Cdkn1c* expression in the placenta (data not shown, from thesis of A.B. Jensen) or in the kidney ($U=11$, $Z=-0.213$, $p=0.831$). Additionally, this was not a general neural response to gestational adversity as a high fat diet did not affect *Cdkn1c* ($t(20)= -0.269$, $p=0.791$). Gestational high fat diet did, however, cause an increase in neural *Igf2* expression ($t(19)= 3.938$, $p=0.001$) and *Dlk1* ($t(20)= 2.143$, $p=0.045$) (Figure 7.2B). See table 7.1 for full details of imprinted genes after prenatal dietary alterations.

Figure 7.2: Selected imprinted gene expression relative to the geometric mean of two housekeeping genes. Shown is fold change in expression compared to basal diet condition after maternal (A) low protein or (B) high fat diet. Genes which were significant after adjustment for multiple comparisons are indicated by asterisks. Data shown is mean ± SEM. * p<0.05 ** p<0.01

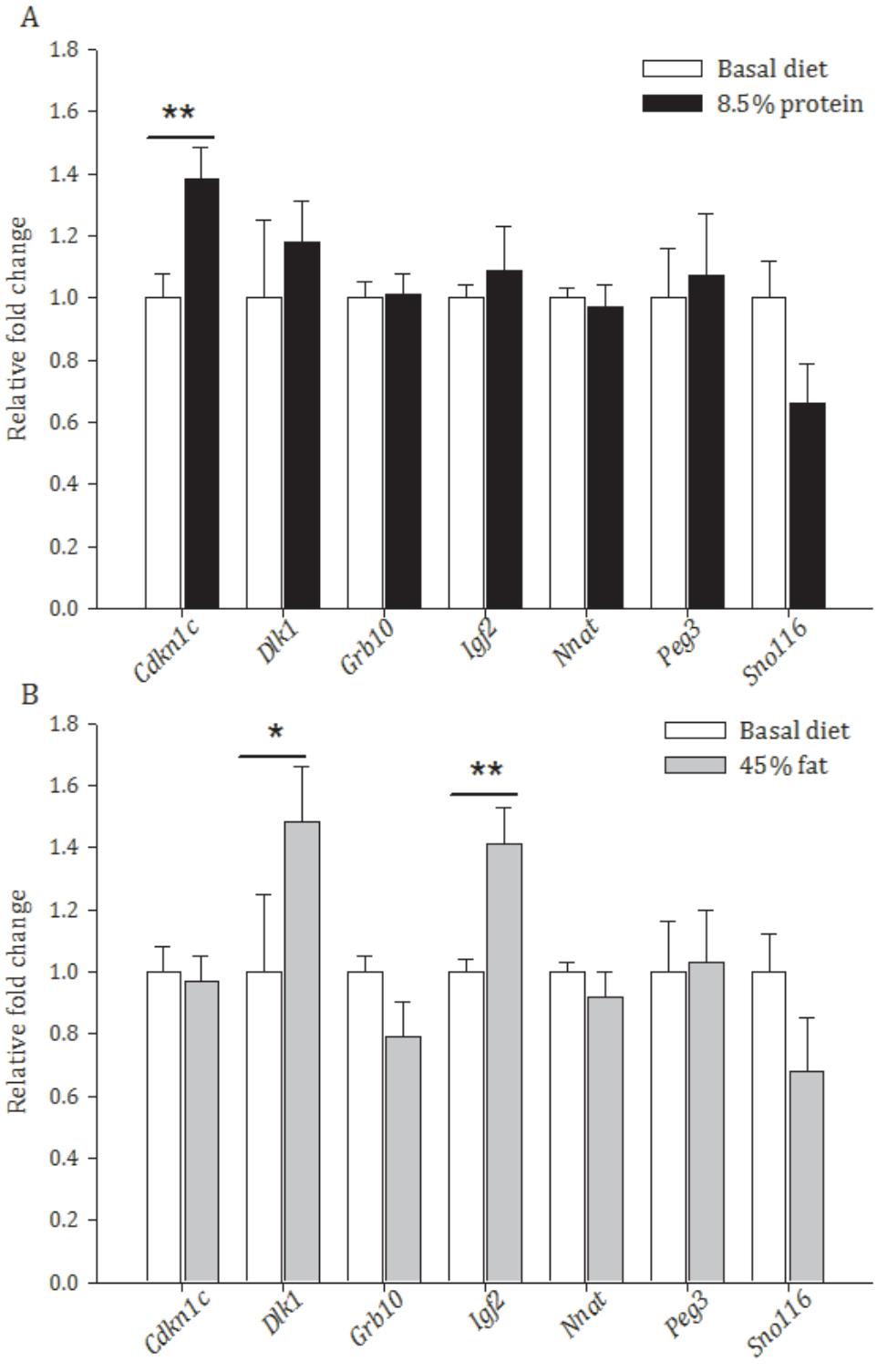
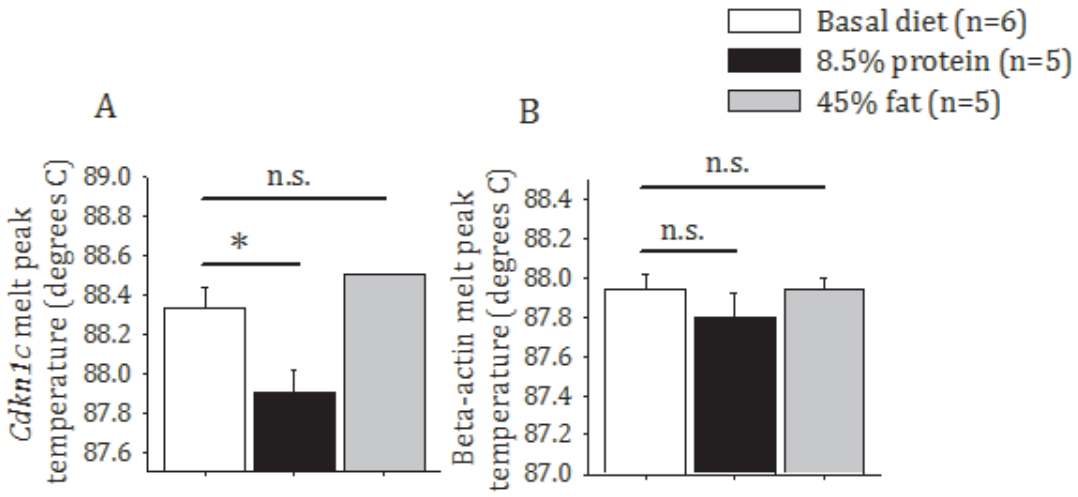


Table 7.1: Table showing t- statistic and significance level of each imprinted gene for the two prenatal conditions vs. basal diet condition. In bold are results that reach nominal significance of α=0.05, Bonferroni adjusted α=0.007.				
Gene name	Basal diet vs. low protein		Basal diet vs. high fat	
	t statistic	p	t statistic	p
Cdkn1c	**t(22)= -3.236**	**0.004**	t(20)= -0.269	0.791
Dlk1	t(22)= -1.305	0.205	**t(20)= 2.143**	**0.045**
Grb10	t(21)= -0.055	0.957	t(19)= -1.627	0.12
Igf2	t(21)= -0.556	0.584	**t(17)= 3.938**	**0.001**
Nnat	t(21)= 0.353	0.728	t(19)= -0.872	0.394
Peg3	t(22)= -0.317	0.754	t(20)= 0.113	0.911
Snord116	t(22)= 1.873	0.074	t(20)= -1.539	0.139

7.3.3 Parental allelic contribution to Cdkn1c expression

There was a significant difference between the peak melt temperature of *Cdkn1c* after an *in utero* exposure to a low protein diet compared to the basal diet condition (U=4, Z=-2.106, p=0.035) (Figure 7.3A). There was no difference in the peak melt temperature between the basal condition and *in utero* exposure to a high fat diet (U=12, Z=-1.483, p=0.138) (Figure 7.3A). Importantly, there was no difference in the peak melt temperature between gestational low protein diet and the basal condition in *βactin* (U=10.5, Z=-0.898, p=0.369) (Figure 7.3B).

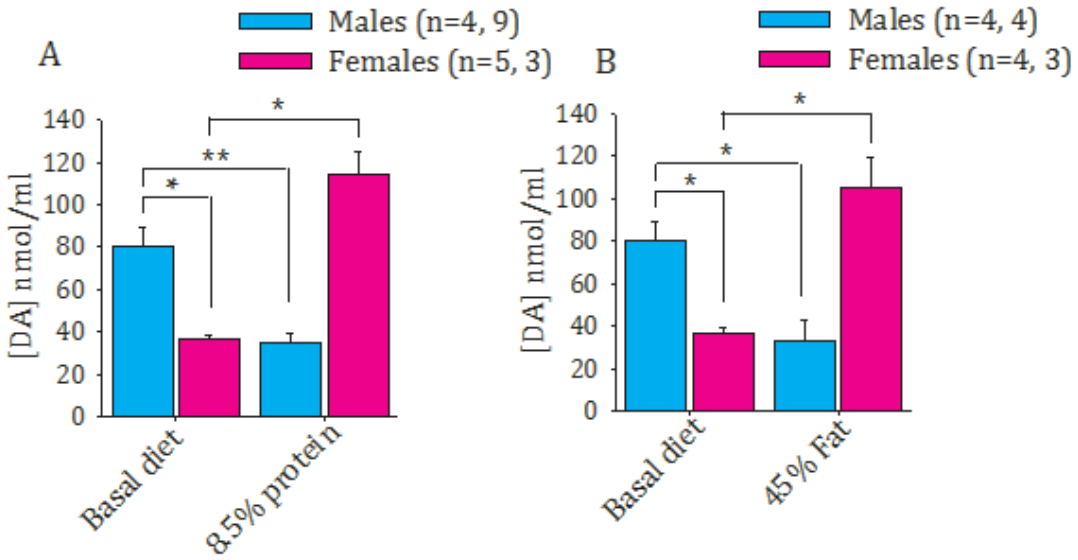
Figure 7.3: The peak melt temperature of the qPCR product of (A) *Cdkn1c* but not (B) *βactin* changes after prenatal low protein diet. Data shown is mean ± SEM. * p<0.05



7.3.4 Sexually dimorphic consequences for dopaminergic system after gestational adversity

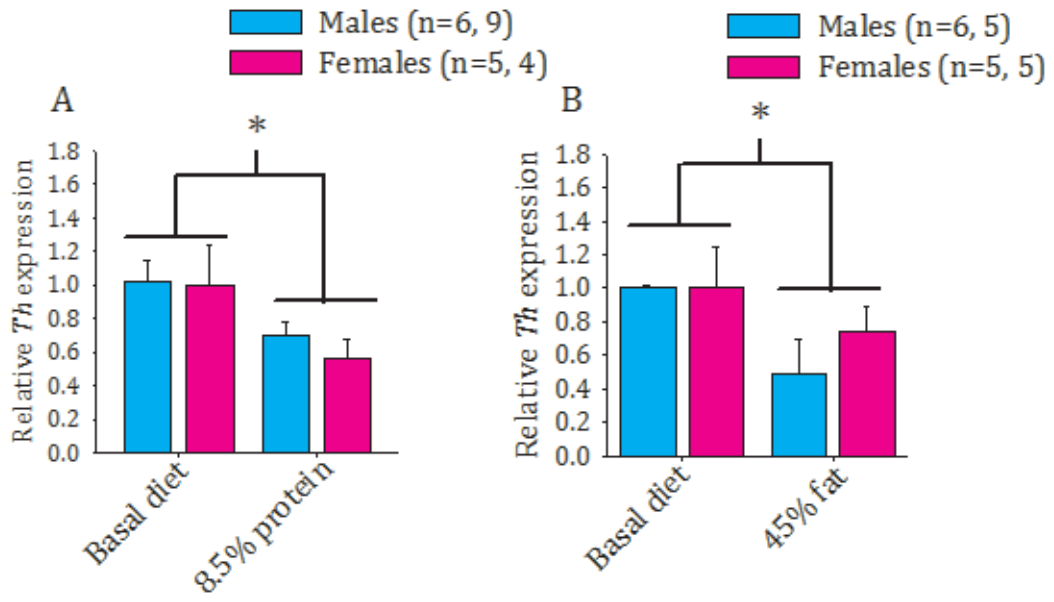
In the basal condition, males had significantly higher levels of dopamine in the fore- and midbrain than females (U=0, Z=-2.449, p=0.014) (Figure 7.4A, B). However, after *in utero* exposure to both high fat and low protein there were consistent, sexually dimorphic effects on levels of dopamine. In females dopamine significantly increased as a result of exposure to a low protein (U=0, Z=-2.121, p=0.034) (Figure 7.4A) or high fat (U=0, Z=-2.236, p=0.025) (Figure 7.4B) maternal diet. The opposite was observed in male offspring, with a significant decrease in dopamine after *in utero* exposure to a maternal diet low in protein (U=0, Z=-2.777, p=0.005) (Figure 7.4A) or high in fat (U=0, Z=-2.309, p=0.021) (Figure 7.4B) in the fore- and mid-brain at E18.5.

Figure 7.4: Fore- and mid-brain dopamine concentration changed in a sex dependant manner after prenatal (A) low protein or (B) high fat diet at E18.5. Data shown is mean ± SEM. n=(number in basal, number in test condition) * p<0.05, **p<0.01



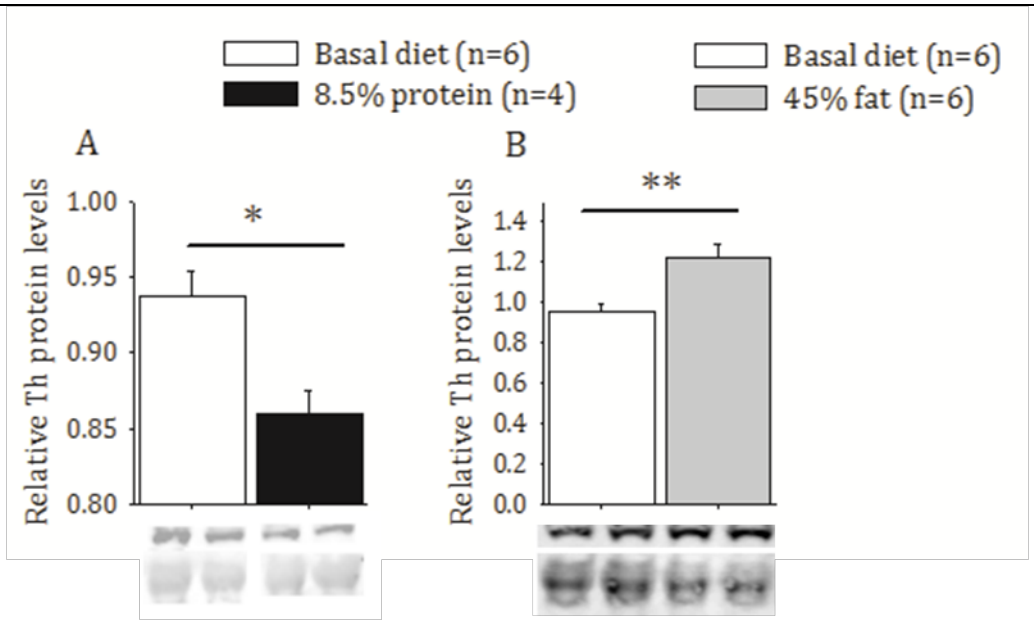
The changes in dopamine were accompanied by a decrease in expression of *Th*, after both maternal low protein (t(22)= -2.404, p=0.025) (Figure 7.5A) and maternal high fat (t(19)= -2.51, p=0.021) (Figure 7.5B) diet compared to the basal diet condition. This trend occurred in both sexes across both conditions, though when analysed individually, only the change in *Th* in male offspring exposed to a gestational low protein diet was significant (t(13)= -2.413, p=0.031). There was no change in the levels of *Dat* or *Drd1* after maternal low protein (*Dat*: U=56, Z=-0.441, p=0.659; *Drd1*: U=67, Z=-0.261, p=0.794) or high fat diet (*Dat*: U=31, Z=-0.839, p=0.402; *Drd1*: U=41, Z=-0.986, p=0.324).

Figure 7.5: Fore- and mid-brain *Th* expression is reduced after prenatal (A) low protein or (B) high fat diet. Data shown is mean ± SEM. n=(number in basal, number in test condition) * p<0.05,



Additionally, there was a small but significant decrease in Th protein levels relative to βactin, after exposure to a maternal low protein diet (U=2, Z=-2.132, p=0.038) (Figure 7.5A). There were no changes in the protein levels of either Drd1-like receptors (t(8)=-0.882, p=0.430) or Drd2-like receptors after maternal low protein diet(t(8)=-0.555, p=0.594). There was a significant increase in the levels of Th relative to βactin after exposure to a maternal high fat diet during gestation (t(10)=-3.292,p=0.008) (Figure 7.6B). Further analysis revealed this to be as a result of the significant increase in Th levels in females (t(5)=-5.503,p=0.005), which was not the case in males (t(5)=-1.274,p=0.272). Neither Drd1-like nor Drd2-like receptors were altered after high fat diet (Drd1-like: t(10)=-0.805, p=0.440; Drd2-like: t(10)=-2.233, p=0.05).

Figure 7.6: Fore- and mid-brain Th protein level (A) decreased after prenatal low protein and (B) increased after prenatal high fat diet. Data shown is mean ± SEM. Shown below graphs are two representative lanes from each condition showing Th and respective βactin loading control. * p<0.05, **p<0.01



7.4 Discussion

This chapter concerns the effects of maternal diet during pregnancy on offspring imprinted gene expression and neural development. These data showed that imprinted genes as a group were generally sensitive to the maternal environment. However, subsets of imprinted genes did respond to the maternal environment, and in the case of *Cdkn1c* this was in an organ specific manner. Maternal high fat and low protein diets were associated with sexually dimorphic changes to the dopaminergic system of the offspring at E18.5. Both maternal conditions decreased dopamine levels in the fore- and mid-brain of male offspring but increased dopamine levels in the female offspring. This was accompanied by changes in levels of Th, at both mRNA and protein levels.

7.4.1 Imprinted genes and maternal diet

Altering the maternal diet had no significant effect on the weights of the offspring or the placenta at E18.5; this was similar to that reported previously for prenatal low protein diet (Vucetic et al., 2010b) but not high fat (Vucetic et

al., 2010a). After exposure to a maternal diet low in protein there was a significant increase in *Cdkn1c* in the fore- and mid-brain of offspring at E18.5, with no change in *Igf2* levels. After maternal high fat diet *Cdkn1c* levels were unchanged. However, there was a significant increase in levels of *Igf2* and *Dlk1*, though the latter result did not survive correction for multiple testing. The relative specificity of alterations in imprinted gene expression in response to a given environmental insult, in addition to the observed tissue specificity with regards to *Cdkn1c*, implies a reactive, rather than passive, response to the maternal environment. This has been alluded to previously (Charalambous et al., 2007; Radford et al., 2011; Radford et al., 2012) for the liver and placenta but this is the first such observation for the brain.

Parental allelic contribution to the *Cdkn1c* PCR product was determined utilising the presence of SNPs within the paternally inherited allele which changed the PCR product nucleotide composition and, therefore, the product melt temperature. The peak melt temperature of a PCR product is dependent on the nucleotide sequence of which it is composed. Differences in peak melt temperature between conditions are considered indicative of a change in product nucleotide composition and, therefore reactivation of the SNP containing, normally silent paternal allele. This implied that the observed increase in *Cdkn1c* expression after maternal low protein diet was due to an increased contribution of the paternal allele. Such analysis was not carried out for *Igf2* as is not thought to be imprinted in the brain (Ohlsson et al., 1994; Pham et al., 1998) and therefore investigation of parental allelic contribution to expression would not be particularly informative. The mechanism through which there may be a release of monoallelic expression was not directly investigated here. However, in the case of *Cdkn1c,* after maternal low protein diet it is possible that the S-Adenosyl methionine (SAM) poor environment resulted in a shortage of methyl donors and, therefore, a failure to correctly establish the correct methylation at the *Cdkn1c* DMR. This does not account for the observed tissue or imprinted gene specificity, however, highlighting the likelihood of the increase in *Cdkn1c* expression as being an active response to the prenatal environment.

Cdkn1c, Igf2 and *Dlk1* have all been associated with the development of the dopaminergic system (Joseph et al., 2003; Christophersen et al., 2007; Bauer et al., 2008; Freed et al., 2008; Jacobs et al., 2009; Vazin et al., 2009). Additionally, all three of these genes have been reported to be sensitive to the prenatal and early post natal environment in a number of studies (Kwong et al., 2006; Gallou-Kabani et al., 2010; Gong et al., 2010; Vucetic et al., 2010b; Drake et al., 2011; Lin et al., 2012; Radford et al., 2012; Claycombe et al., 2013; King et al., 2013; Sferruzzi-Perri et al., 2013; Susiarjo et al., 2013; Jensen Peña et al., 2014) . While the majority of studies examining this phenomenon have reported alterations outside the CNS, at least one study has reported both neural *Igf2* and *Cdkn1c* to change in response to the *in utero* environment (Vucetic et al., 2010b). Changes in *Igf2* in response to maternal low protein diet during pregnancy were not observed here; however this may be due to the time of sampling (E18.5 versus adult).

While it did not maintain significance after correction of multiple testing, there was a nominally significant increase in *Dlk1* in the embryonic fore- and mid-brain after a prenatal high fat diet. Differences in *Dlk1* expression in the brain after a high fat diet have not been previously reported, however, *Dlk1* has previously been shown to be altered in the placenta after a prenatal high fat (Gallou-Kabani et al., 2010) and a prenatal high fat- high sugar diet (Sferruzzi-Perri et al., 2013). *Dlk1* expression profile in the adult rodent in monoaminergic neurons of the mid- and hindbrain, the ventral tegmental area (VTA), substantia nigra (SN), locus ceruleus (LC) and raphe nuclei (Jensen et al., 2001), implies a role for this gene in the dopaminergic system. Similar to *Cdkn1c*, *Dlk1* is a target of Nurr1 *in vivo* (Jacobs et al., 2009), peaks in the developing midbrain at E13.5 (Christophersen et al., 2007) and is expressed in the developing embryonic dopaminergic neurons (Bauer et al., 2008; Jacobs et al., 2009). The function of *Dlk1* appears to be promote the proliferation of dopaminergic (Christophersen et al., 2007; Bauer et al., 2008) and serotoninergic neurons (Bauer et al., 2008) and to prevent premature *Dat* expression in these neurons (Jacobs et al., 2009). While there was no significant changes in *Dat* expression in this study (data not

shown) the difference in *Th* and dopamine imply that the increased expression of *Dlk1* may be functionally relevant. These data highlight the sensitivity and specificity of the response of a subset of imprinted genes to changes in the prenatal environment.

7.4.2 Sexually dimorphic consequences for the dopaminergic system

The observed changes in the dopaminergic system were sexually dimorphic, a finding not observed in changes in imprinted gene expression. While these may be separate observations, it is also possible that the alterations in imprinted gene regulation occurs upstream of the dopaminergic changes and the sex specific effects are as a result of interaction with other secondary factors, such as the sex hormones. This is particularly plausible as the changes in *Th* at the mRNA level were also independent of sex, emphasising the possibility for factors external to the dopaminergic system to be influencing levels of this transmitter.

After both test diet conditions there was a reversal in the direction in the sexual dimorphism in levels of dopamine, with females being hyperdopaminergic with respect to male littermates. Sexual dimorphism in the dopaminergic system has been well established although this work has almost exclusively been carried out postnatally. It is well known that, in females, estrogen can potentiate the effect of manipulations of the dopaminergic system, for example amphetamine stimulated dopamine release (Castner et al., 1993). Additionally females are more sensitive to the effects of amphetamine on locomotor activity (Castner et al., 1993; Becker, 1999)(becker 1999) and cocaine (Walker et al., 2001). Estrogen fluctuates across the female menstrual cycle, peaking immediately before, and again after, ovulation in humans and between di- and pro-estrus in rats (Bobzean et al., 2014). Estrogen is required, at least in females, for survival of VTA DAergic neurons (Johnson et al., 2010b). Aromatase, the estrogen synthesising enzyme which converts testosterone to estrogen, is expressed in neurons in the developing mouse hypothalamus and cortex (Beyer et al., 1994b). However, this enzyme is more active in the male murine hypothalamus

at E17 and P2 than female (Beyer et al., 1994a). Prenatally, male rat fetuses have been shown to have more dopamine than females at E21 (Izvolskaia et al., 2009). Differences in the number of Th immuno-reactive cells at in rodent midbrain may underlie these sex differences (McArthur et al., 2006), though this evidence is conflicting was not significant (Reisert et al., 1990; Lieb et al., 1996). However, in female rats a significantly greater proportion of mesocortical projections were dopaminergic compared to males (Kritzer and Creutz, 2008). Nonetheless, these sex differences are maintained into adulthood with adult male mice having increased dopamine compared to females in the accessory olfactory bulb, medial pre-optic area and striatum (Wersinger and Rissman, 2000).

Although there was an increase in fore- and midbrain dopamine concentration in females after both low protein and high fat diet, there was a concomitant decrease in *Th* at the mRNA level for both males and females after both dietary conditions. Given the relatively crude dissection, it is possible that some molecular information was lost due to presence of unaffected brain regions in sample. However, it seems more plausible that this was due to underlying hormonal differences, particularly given the differences in the aromatase activity in the hypothalamus at this age between male and female rodents (Beyer et al., 1994a) and this time point, which is a critical period for the generation of a sexually dimorphic brain (Rhees et al., 1990). Additionally, maternal nutritional restriction during gestation leads to early life increases in progesterone and earlier puberty in rats (Sloboda et al., 2009). This implies the pre-existing sex difference and environmentally induced difference could potentially interact, producing sexually dimorphic responses in the offspring. In the case of a gestational low protein diet, the decrease in *Th* at the mRNA level was repeated at the protein level. However after a prenatal high fat diet there was an increase specifically in females of Th protein, compared to the basal diet condition, as would be expected given the increase in dopamine concentration. This discrepancy between mRNA and protein levels relative to the basal diet condition in females after prenatal high fat diet may be due to differences in

protein stability, though this is not possible to determine from the data presented here.

These findings implicate imprinted genes as a site upon which gestational adversity may act, in an organ and tissue, but not necessarily sex, specific manner. The imprinted genes that were altered after prenatal low protein (*Cdkn1c*) or high fat diet (*Dlk1* and *Igf2*) functionally converge on the dopaminergic system. Potentially as a directly downstream consequence of aberrant imprinted gene expression, there was a sexually dimorphic dysregulation of genes and proteins related to dopamine synthesis. This has the effect of a reversal in the direction of sexual dimorphic nature of neural dopamine concentration. Importantly, this is the first report of the prenatal consequences of gestational adversity on both neural imprinted genes and the dopaminergic system. This allows exclusion of any possible confounding interaction with the postnatal environment. These findings have important implications for the postnatal consequences of gestational adversity, highlighting the importance of imprinted gene dosage and the *in utero* environment for appropriate brain development.

Chapter 8: General discussion

Imprinted genes are a subset of functionally monoallelic genes present in mammals and some flowering plant species. Appropriate dosage of these genes has been shown to be crucial for normal development (Kaufman et al., 1977; Surani and Barton, 1983; McGrath and Solter, 1984b; Surani et al., 1984; Thomson and Solter, 1988). Expression levels of these genes have also been shown to be sensitive to the *in utero* and early post-natal environment (Table 1.1) (Gallou-Kabani et al., 2010; Vucetic et al., 2010b; Radford et al., 2012; McNamara and Isles, 2014). Functionally these genes frequently converge on specific biological process including, but not limited to, placental function (Frost and Moore, 2010), metabolism (Smith et al., 2006) and behaviour (Wilkinson et al., 2007). *Cdkn1c* is a maternally expressed imprinted gene and the focus of this thesis. A BAC over expression model of this gene (*Cdkn1c*$^{\mathrm{BACx1}}$ line) (John et al., 2001) was characterised for the first time with respect to neuronal development and behaviour. In addition, the sensitivity/resilience of this gene, and other imprinted genes, to a suboptimal prenatal diet was examined. The consequences for dopaminergic system development were also described.

8.1 Summary of main findings

A summary of the main molecular (Table 8.1) and behavioural (Table 8.2) results can be found below.

8.1.1 Cdkn1c^{BACx1} and the dopaminergic system

This work focussed *Cdkn1c* dosage and consequences of a two fold increase in expression on the dopaminergic and serotonergic systems. Overall, there were relatively subtle but functionally significant change in these neurotransmitter systems in the brain of adult male mice over expressing *Cdkn1c*. Changes in tissue neurotransmitter levels were observed in the frontal cortex, dorsal striatum, ventral striatum and hypothalamus. There was a decrease in expression of both *Drd1* and *Drd2* receptors in the frontal cortex, the target of the mesocortical dopaminergic system, originating in the VTA. In adult rats virally mediated over expression of *Drd1* in prefrontal dopaminergic neurons caused an increase in sucrose and saccharin preference and an increased motivation to work for cocaine in a progressive ratio task (Sonntag et al., 2014).

In this thesis, it was shown that *Cdkn1c*^{BACx1} animals have a decreased perceived palatability for sucrose. In addition, *Cdkn1c*^{BACx1} animals had a heightened motivational drive to work to obtain a sucrose reward in a PR task. Therefore, it is conceivable that a decreased abundance of *Drd1* receptors in the frontal cortex may be contributing, at least in part, to this phenotype. The decrease in receptor abundance may be a developmental compensatory mechanism to hyper-dopaminergic input from the midbrain. Following activation by dopamine both Drd1 and Drd2 and internalised with selective Drd2 degradation *in vitro* (Bartlett et al., 2005). Repeated cocaine administration, and therefore, increased extracellular dopamine, similarly causes degradation selectively of Drd2 receptor *in vivo* in the striatum (Thompson et al., 2010; Madhavan et al., 2013), linking an increase in dopaminergic activity to a selective decrease in Drd2. Genetically hyper-dopaminergic mice (following ablation of *Dat* gene) displayed decreased abundance of Drd1 and Drd2 in the basal ganglia and ventral midbrain (Giros et al., 1996). In the case of Drd1, this was reversed by administration of 6-hydroxydopamine (Dumartin et al., 2000), linking the receptor decrease directly to the altered dopaminergic state. Finally, repeated i.p. methamphetamine administration has been shown to cause a decrease in Drd1 and Drd2 signalling in the frontal cortex (Mizoguchi et al., 2007). Together, these data suggest that an altered dopaminergic drive onto the frontal cortex can result in decreased receptor abundance in this region, though further work is required to characterise this in *Cdkn1c*^{BACx1} animals.

Over expression of *Cdkn1c* resulted in a deficit in PPI. This hypothesised endophenotype has been linked to schizophrenia and is apparent in drug naive, first episode patients with schizophrenia (Düring et al., 2014) and non-psychotic individuals with an ultrahigh risk of developing psychosis (De Koning et al., 2014). PPI modulation has been linked to the dopaminergic system as anti-psychotic drugs, with antagonistic activity at Drd2-like receptors such as haloperidol, have been shown to reverse a PPI deficit (Tournier and Ginovart, 2014). However, in this study selective Drd2/Drd3 receptor blockade did not reverse this deficit in patients, though symptom severity was reduced (De Koning et al., 2014), indicating other neurotransmitters system may

additionally regulate this behaviour. The serotonergic system has been implicated in underlying variability in this behaviour. Agonism of serotonin 2a-receptor ($5HT_{2a}$ R) has been shown to reduce PPI in healthy subjects (Vollenweider et al., 2007). Given the observed changes in tissue levels of serotonin in the frontal cortex, dorsal and ventral striatum in *Cdkn1c*^{BACx1} animals, it is not possible to assign the deficit in PPI to changes in a given neurotransmitter system. Nonetheless, this work demonstrated the importance of *Cdkn1c* dosage in development of correct sensorimotor gating and potentially other biomarkers of psychotic illness not examined in this thesis.

In the dorsal striatum, animals over expressing *Cdkn1c* were found to have an increased tissue level of dopamine. This was emphasised by a significant increase in *Dat* mRNA levels compared to wt littermates and an increase in Th immuno-reactivity, in this region and in the ventral striatum. This co-occurred with an increased motivational drive to obtain a sucrose reward while finding the sucrose less palatable compared to wt littermates. Importantly, this increase in motivational drive was apparent at eqi-palatable concentrations of sucrose. Additionally, though BP remained elevated with respect to wt littermates, *Cdkn1c*^{BACx1} animals had a significant reduction in BP when working to obtain non calorific saccharin. This implies *Cdkn1c*^{BACx1} animals were more motivated by the calorifically rewarding aspects of sucrose, compared to its palatability, than wt littermates. This may have implications for the human imprinting disorder, SRS, which is associated with increased *CDKN1C*. Some SRS children are reported as being fussy eaters (Blissett et al., 2001). It is possible that these children are not 'fussier' *per se*, but do not perceive food to be as palatable. Importantly calorie intake is no different in these children (Blissett et al., 2001), similar to that observed in *Cdkn1c*^{BACx1} animals. Additionally IUGR preterm infants at 16 days display decreased hedonic responding to a sucrose solution (Ayres et al., 2012).

The observed dissociation between reward 'liking' and reward 'wanting' is not without precedence in genetically hyper-dopaminergic animals (Pecina et al., 2003; Drew et al., 2007). The incentive sensitization theory of addiction

concerns the dissociation between the hedondic value of a reinforce and its motivational properties (Robinson and Berridge, 1993, 2008). This theory posits that, in a subset of individuals using drugs of abuse, the neurobiological perceived 'importance' of the drug (i.e. how much it is 'wanted') is heightened excessively, through associative learning of drug related cues etc., resulting in habitual and abusive drug taking. The theory further postulates that this occurs independent of the hedonic properties of the drug, and that, in fact, subjective pleasure decreases with repeated administration (Robinson and Berridge, 1993). This is reminiscent of *Cdkn1c*^{BACx1} animals' responding to sucrose reinforcer. This finding is limited, in that it reflects behaviour towards a natural, not classically considered addictive, substance. In addition to a dissociation between reinforce 'wanting' and 'liking', animals over expressing *Cdkn1c* were hypersensitive, behaviourally and neurobiologically, to a sub-stimulatory dose of amphetamine. The ventral striatum of these animals, which contains the nucleus accumbens, displayed increased reactivity to an i.p. amphetamine injection, compared to wt littermates and the control *Cdkn1c*^{BACLacZ} animals. This is similar to previous work on cocaine responsivity in adult male offspring exposed to a prenatal low protein diet, also associated with increased *Cdkn1c* dosage (Vucetic et al., 2010b). Taken together, these data imply that increased *Cdkn1c* dosage primes an 'addictive-like' state.

Table 8.1: Summary of main findings from Chapter 3: Molecular characterisation of *Cdkn1c*^{BACx1} adult brain. (*Cdkn1c*^{BACLacZ} did not differ from wt littermates in any of these measures). ↑ *Cdkn1c*^{BACx1} increased compared to wt littermates. ↓ *Cdkn1c*^{BACx1} decreased compared to wt littermates. ↔ *Cdkn1c*^{BACx1} no change from wt littermates.

	Frontal cortex	**Dorsal striatum**	**Ventral striatum**	**Hypothalamus**
Drd1	↓	↔	↔	↔
Drd2	↓	↔	↔	↔
***Th* (mRNA)**	↔	↔	↔	↔
Th (immuno reactivity)	**n/a**	↑	↑	**n/a**
Dat	↔	↑	**n/a**	**n/a**
[Dopamine]	↔	↑	↔	↔
[Serotonin]	↔ (↓)	↑	↑	↔
[DOPAC]	↔	↔	↔	↓
[5HIAA]	↔	↔	↔	↓

Table 8.2: Summary of main findings from Chapters 4-6 concerning the behavioural characterisation of *Cdkn1c*^{BACx1} line. (*Cdkn1c*^{BACLacZ} did not differ from wt littermates in any of these measures). ↑ *Cdkn1c*^{BACx1} increased compared to wt littermates. ↓ *Cdkn1c*^{BACx1} decreased compared to wt littermates. ↔ *Cdkn1c*^{BACx1} no change from wt littermates.

Behaviour type	**Test**	**Direction of effect of *Cdkn1c*^{BACx1} genotype**
Motoric- Chapter 4	*Rotarod*	↔
	Locomotor activity (beam breaks)	↓
	Response to amphetamine	↑
Anxiety- Chapter 4	*Open field*	↔
	Elevated plus maze	↔
Reward related - Chapter 5	*Motivation in a PR task*	↑
	Hedonic responding	↓
Sensorimotor gating- Chapter 4	*Baseline startle*	↓
	Pre-pulse inhibition	↓
Social- Chapter 6	*Tube test (stranger)*	↑
	Social stability	↓

An environmental prenatal/early life role for increased *Cdkn1c* expression has previously been described (Vucetic et al., 2010b; Jensen Peña et al., 2014). This thesis replicated previous findings with respect to a prenatal low protein diet, and expanded them, attributing the increase in *Cdkn1c* to expression from the paternal allele. Paternal allele expression has also been observed in some other tissues in response to low protein diet (Van De Pette, in preparation). This thesis examined the prenatal consequences of a suboptimal maternal gestational diet on additional neural imprinted gene expression and the dopaminergic system. A prenatal low protein diet has previously been linked to alterations in dopamine related behaviours (Vucetic et al., 2010b; Reyes-Castro et al., 2011), with a sexually dimorphic presentation (Reyes-Castro et al., 2011; Reyes-Castro et al., 2012a; Reyes-Castro et al., 2012b). Sexually dimorphic consequences for the dopaminergic system were observed in this thesis. There

was a decrease and increase in the levels of dopamine in males and females, respectively, in the fore- and mid-brain at E18.5 after prenatal low protein or high fat diet. Few studies have examined the consequences of a prenatal low protein diet on the offspring prenatally. Studies that have investigated prenatal consequences have focussed on opioid and feeding related signatures (Terroni et al., 2005) and structural and morphological differences (Wainwright and Stefanescu, 1983; Gressens et al., 1997).

Genetic over expression of *Cdkn1c* recapitulated a subset of the behavioural and molecular signatures associated with a maternal low protein diet during pregnancy and lactation, including increased *cfos* reactivity to a stimulant in the ventral striatum (Vucetic et al., 2010b), decreased sucrose preference/ perceived palatability (Vucetic et al., 2010b) and other, anhedonia-associated behaviours (Godoy et al., 2013), inappropriate social behaviours (Almeida et al., 1996), increased responding for a food reward (Tonkiss et al., 1990), increased straital *Th* and *Dat* (Vucetic et al., 2010b) and regional specific increases in dopamine (Vucetic et al., 2010b). Furthermore, though not assessed in this study, additional *Cdkn1c* regulated processes have been shown to be altered in the offspring following prenatal and lactational low protein diet. These include adult neural stem cell regulation (Godoy et al., 2013), neuronal migration and maturation (Morgane et al., 1993) and correct corticogenesis (Gressens et al., 1997).

Adult males exposed to a prenatal and lactational low protein diet have increased dopamine levels in the prefrontal cortex and increased *Th* in the striatum (Vucetic et al., 2010b), similar to *Cdkn1c*$^{\mathrm{BACx1}}$ adult males. However, at a prenatal time point male foetuses exposed to a low protein environment have decreased dopamine and *Th* in the fore- and mid-brain compared to fetuses fed a basal diet. This developmental switch from a prenatal hypo-dopaminergic state to a postnatal hyper-dopaminergic state after prenatal adversity has been shown for prenatal alcohol exposure (Rathbun and Druse, 1985; Sari et al., 2010). This highlights the priming effect of prenatal environmental insults and the potential for interaction with the post-natal environment. This is relevant

given the observation that raising a litter exposed to a prenatal low protein environment alters dam mothering behaviour, independent of dam gestational diet (Galler and Tonkiss, 1991). The results of this thesis suggest that it may be possible to attribute elements of the alterations in reward processing following a prenatal low protein diet specifically to the over expression of *Cdkn1c*.

It is not possible to establish a direct link between dopamine associated reward related behaviours observed after prenatal high fat diet (Teegarden et al., 2009; Vucetic and Reyes, 2010; Naef et al., 2011; Vucetic et al., 2012; Grissom et al., 2013) to over expression of the imprinted gene *Igf2* and *Dlk1* from the work in this thesis. Mechanistically these two systems are linked given the role *Igf2* and *Dlk1* in promoting development of dopaminergic neurons of the midbrain (Christophersen et al., 2007; Bauer et al., 2008; Freed et al., 2008; Jacobs et al., 2009; Vazin et al., 2009). To separate correlation from causation it would be necessary to carry out similar experiments on transgenic animals over expressing either *Igf2* or *Dlk1* individually, or both in combination.

8.1.2 Cdkn1c and social stability

Presence of an animal over expressing *Cdkn1c* in a social group caused a destabilisation of the social hierarchy, and resulted in in more frequent rank changes and a consequence for individual fitness. Both the dopaminergic and serotonergic system have been implicated in modulating social behaviours. Dat blockade, and increased dopamine availability, in the mouse from P22-41 increased adult aggressive behaviours (Yu et al., 2014). Optogeneic stimulation of VTA dopamingeric neurons increased aggressive behaviours (Yu et al., 2014) and dopamine in the pre-frontal cortex and the ACC was shown to be elevated in intruder rats in the presence of an aggressive resident (Tidey and Miczek, 1996). This could be a recapitulation, in *Cdkn1c*$^{\mathrm{BACx1}}$ animals, of an altered dopaminergic drive from the mesocortical pathway. A neuronal knockout of Drd2 receptor reduced the levels of excreted major urinary proteins in adult mice. Furthermore, urine from a knock out male failed to induce aggression towards an intruder (Noain et al., 2013). Greater activity onto Drd2 receptors, therefore, could potentially provoke more frequent challenges. Additionally,

Drd1 activation by a selective agonist, but not Drd2 inhibition, enhances social recognition (Loiseau and Millan, 2009). Drd1 levels in the frontal cortex dynamically respond in males to repeated defeats in antagonistic social interactions (Avgustinovich and Alekseyenko, 2010). Therefore, the decreased *Drd1* mRNA abundance in the *Cdkn1c*^{BACx1} animals may underlie the abnormalities in social behaviours.

The serotonergic system has also been implicated as a regulator of aggression and violence (Raleigh et al., 1991; Edwards and Kravitz, 1997; Krakowski, 2003). As a consequence of the complexity of serotonergic receptor pharmacology it is non-trivial to ascribe a definitive role for this neurotransmitter in mediating aggressive behaviours. Increased serotonergic functioning has been linked to increased aggressiveness of a dominant status. A study in adult vervet monkeys showed that serotonin can bidirectionally influence dominancy acquisition, serotonin agonists promoted dominant status and serotonin antagonists promoted subordinate status (Raleigh et al., 1991). A polymorphism in the serotonin (and dopamine) metabolising enzyme *monoaminoxidase A* promoter, which results in decreased promoter activity and therefore increased transmitter availability, was linked to increased aggressiveness in adult men (Manuck et al., 2000). However, serotonin has also been implicated in promoting a subordinate state. Among males alone, aggression was negatively correlated with $5HT_{2C}$ receptor abundance (Soloff et al., 2010). Serotonin transporter blockade from P22-41 in the mouse reduced aggressive behaviours (Yu et al., 2014). Therefore, while it is apparent that the role of serotonin in social behaviour is likely dependent on the receptor subtype activated, it is possible that the perturbed social stability in groups containing *Cdkn1c*^{BACx1} animals is due to the alterations in this neurotransmitter.

8.1.3 Cdkn1c^{BACLacZ} line

With the exception of consumption in the lick microstructure analysis, none of the behavioural or molecular analyses revealed a difference between *Cdkn1c*^{BACLacZ} animals and their wt littermates. These animals have wt expression levels of *Cdkn1c*. As illustrated in Figure 2.1C in this work, these

animals carry an additional copy of the imprinted genes *Phlda2* and *Slc22a18*. *Phlda2* regulates junctional zone of the placenta with contains the glycogen cells and the placental endocrine cells (Tunster et al., 2010). On a 129/Sv background this transgene drives a progressive slow down in fetal growth (Tunster et al., 2010; Tunster et al., 2014). This was not the case for a single copy transgene on the BL6 background (Tunster et al., 2014), the model used in this study. The programming effects of altered placental imprinted gene expression on adult behaviour have been demonstrated in relation to *Igf2* (Mikaelsson et al., 2013). Behavioural tasks carried out, in this thesis, were selected as being predominantly dopamine dependant. Programming effects linked to decreased placental *Igf2*, anxiety related behaviour in an open field and elevated plus maze and ASR (Mikaelsson et al., 2013), were not different between *Cdkn1c*$^{\text{BACLacZ}}$ animals and their wt littermates. Further studies are required in order to establish if the *Cdkn1c*$^{\text{BACLacZ}}$ genotype has consequences for brain and behaviour.

8.2 Implications for imprinted genes and an adaptive response to prenatal adversity

Imprinted gene function, by their very nature, is dosage sensitive and, as such, provides a site at which deleterious or advantageous alterations in expression could occur. Given their dosage sensitivity and importance for development, it is possible the tight regulation of these genes would be prioritised in suboptimal conditions, such that the 'status quo' is maintained. Alternatively, these genes could act as a site of 'fine tuning' whereby the developing fetal brain is primed *in utero* for the environment in which its mother exists, and into which it will soon be thrust. Prenatal adversity results in functional alterations in systems which are regulated by imprinted genes including metabolic function (Entringer, 2013), fetal growth (Baibazarova et al., 2013), reproductive function (Sloboda et al., 2009; Perry, 2014) and behaviour (Bale et al., 2010). The seemingly adaptive nature of offspring response to prenatal adversity has been discussed in this thesis in Chapter 1.4.1 (Bateson et al., 2004). The work in this thesis cannot differentiate between an adaptive or passive response in imprinted gene

expression following prenatal adversity. However, the gene, organ and prenatal insult specificity of the alteration in imprinted gene dosage, suggests an adaptive rather than passive cause. An adaptive response of imprinted gene expression suggests a potentially important function for these genes in the plasticity in post natal offspring outcomes to prenatal insults.

This thesis described the consequence of increased *Cdkn1c* dosage on dopaminergic system and on dopamine related, and social, behaviours. The findings in this thesis proposed a role for *Cdkn1c*, and potentially imprinted genes more generally, in an adaptive response to prenatal adversity, via the dopaminergic system. Furthermore, in humans *CDKN1C* dosage may link prenatal malnutrition with subsequent behavioural abnormalities associated with dopamine disregulation, including schizophrenia and addiction (St Clair et al., 2005; Franzek et al., 2008; Malaspina et al., 2008).

Bibliography

Aapola U, Shibuya K, Scott HS, Ollila J, Vihinen M, Heino M, Shintani A, Kawasaki K, Minoshima S, Krohn K (2000) Isolation and Initial Characterization of a Novel Zinc Finger Gene,< i> DNMT3L,</i> on 21q22. 3, Related to the Cytosine-5-Methyltransferase 3 Gene Family. Genomics 65:293-298.

Abel EL (1982) Consumption of alcohol during pregnancy: a review of effects on growth and development of offspring. Hum Biol 54:421-453.

Abel EL (1993) Paternal alcohol exposure and hyperactivity in rat offspring: effects of amphetamine. Neurotoxicology and teratology 15:445-449.

Abel EL (1994) Effects of physostigmine on male offspring sired by alcohol-treated fathers. Alcohol Clin Exp Res 18:648-652.

Aberman JE, Salamone JD (1999) Nucleus accumbens dopamine depletions make rats more sensitive to high ratio requirements but do not impair primary food reinforcement. Neuroscience 92:545-552.

Ager EI, Pask AJ, Gehring HM, Shaw G, Renfree MB (2008) Evolution of the CDKN1C-KCNQ1 imprinted domain. BMC Evol Biol 8:163.

Agis‐Balboa RC, Arcos‐Diaz D, Wittnam J, Govindarajan N, Blom K, Burkhardt S, Haladyniak U, Agbemenyah HY, Zovoilis A, Salinas‐Riester G (2011) A hippocampal insulin‐growth factor 2 pathway regulates the extinction of fear memories. The EMBO journal 30:4071-4083.

Algar E, Dagar V, Sebaj M, Pachter N (2011) An 11p15 imprinting centre region 2 deletion in a family with Beckwith Wiedemann syndrome provides insights into imprinting control at CDKN1C. PLoS One 6:e29034.

Allen ND, Logan K, Lally G, Drage DJ, Norris ML, Keverne EB (1995) Distribution of parthenogenetic cells in the mouse brain and their influence on brain development and behavior. Proceedings of the National Academy of Sciences 92:10782-10786.

Almeida SS, Tonkiss J, Galler JR (1996) Prenatal protein malnutrition affects the social interactions of juvenile rats. Physiol Behav 60:197-201.

Anacker C, Zunszain PA, Cattaneo A, Carvalho LA, Garabedian MJ, Thuret S, Price J, Pariante CM (2011) Antidepressants increase human hippocampal neurogenesis by activating the glucocorticoid receptor. Mol Psychiatry 16:738-750.

Andrews SC, Wood MD, Tunster SJ, Barton SC, Surani MA, John RM (2007) Cdkn1c (p57Kip2) is the major regulator of embryonic growth within its imprinted domain on mouse distal chromosome 7. BMC Dev Biol 7:53.

Arakawa H, Arakawa K, Blanchard DC, Blanchard RJ (2007) Scent marking behavior in male C57BL/6J mice: sexual and developmental determination. Behav Brain Res 182:73-79.

Arakawa H, Arakawa K, Blanchard DC, Blanchard RJ (2009) Social features of scent-donor mice modulate scent marking of C57BL/6J recipient males. Behav Brain Res 205:138-145.

Arakawa H, Blanchard DC, Arakawa K, Dunlap C, Blanchard RJ (2008) Scent marking behavior as an odorant communication in mice. Neurosci Biobehav Rev 32:1236-1248.

Arboleda VA, Lee H, Parnaik R, Fleming A, Banerjee A, Ferraz-de-Souza B, Delot EC, Rodriguez-Fernandez IA, Braslavsky D, Bergada I, Dell'Angelica EC, Nelson SF, Martinez-Agosto JA, Achermann JC, Vilain E (2012) Mutations

in the PCNA-binding domain of CDKN1C cause IMAGe syndrome. Nat Genet 44:788-792.
Arima T, Kamikihara T, Hayashida T, Kato K, Inoue T, Shirayoshi Y, Oshimura M, Soejima H, Mukai T, Wake N (2005) ZAC, LIT1 (KCNQ1OT1) and p57KIP2 (CDKN1C) are in an imprinted gene network that may play a role in Beckwith–Wiedemann syndrome. Nucleic acids research 33:2650-2660.
Arnaud P, Monk D, Hitchins M, Gordon E, Dean W, Beechey CV, Peters J, Craigen W, Preece M, Stanier P (2003) Conserved methylation imprints in the human and mouse GRB10 genes with divergent allelic expression suggests differential reading of the same mark. Hum Mol Genet 12:1005-1019.
Arsalidou M, Duerden EG, Taylor MJ (2013) The centre of the brain: Topographical model of motor, cognitive, affective, and somatosensory functions of the basal ganglia. Hum Brain Mapp 34:3031-3054.
Attia M, Rachez C, De Pauw A, Avner P, Rogner UC (2007) Nap1l2 promotes histone acetylation activity during neuronal differentiation. Mol Cell Biol 27:6093-6102.
Augustine RA, Grattan DR (2008) Induction of central leptin resistance in hyperphagic pseudopregnant rats by chronic prolactin infusion. Endocrinology 149:1049-1055.
Avgustinovich DF, Alekseyenko OV (2010) 3H] SCH 23390 binding in various brain regions of C57BL/6J mice with repeated experience of victory or social defeat in agonistic interactions. Physiological research/Academia Scientiarum Bohemoslovaca 59:455.
Avitsur R, Kinsey SG, Bidor K, Bailey MT, Padgett DA, Sheridan JF (2007) Subordinate social status modulates the vulnerability to the immunological effects of social stress. Psychoneuroendocrinology 32:1097-1105.
Ayres C, Agranonik M, Portella AK, Filion F, Johnston CC, Silveira PP (2012) Intrauterine growth restriction and the fetal programming of the hedonic response to sweet taste in newborn infants. International journal of pediatrics 2012.
Azzi S, Rossignol S, Steunou V, Sas T, Thibaud N, Danton F, Le Jule M, Heinrichs C, Cabrol S, Gicquel C (2009) Multilocus methylation analysis in a large cohort of 11p15-related foetal growth disorders (Russell Silver and Beckwith Wiedemann syndromes) reveals simultaneous loss of methylation at paternal and maternal imprinted loci. Hum Mol Genet 18:4724-4733.
Bahar A, Dudai Y, Ahissar E (2004) Neural Signature of Taste Familiarity in the Gustatory. J Neurophysiol 92:3298-3308.
Baibazarova E, van de Beek C, Cohen-Kettenis PT, Buitelaar J, Shelton KH, van Goozen SHM (2013) Influence of prenatal maternal stress, maternal plasma cortisol and cortisol in the amniotic fluid on birth outcomes and child temperament at 3 months. Psychoneuroendocrinology 38:907-915.
Baik JH (2013) Dopamine signaling in reward-related behaviors. Front Neural Circuits 7:152.
Bale TL, Baram TZ, Brown AS, Goldstein JM, Insel TR, McCarthy MM, Nemeroff CB, Reyes TM, Simerly RB, Susser ES, Nestler EJ (2010) Early life

programming and neurodevelopmental disorders. Biol Psychiatry 68:314-319.
Barker DJ (1995) Fetal origins of coronary heart disease. BMJ: British Medical Journal 311:171.
Barlow DP (1993) Methylation and imprinting: from host defense to gene regulation? Science 260:309-310.
Bartlett SE, Enquist J, Hopf FW, Lee JH, Gladher F, Kharazia V, Waldhoer M, Mailliard WS, Armstrong R, Bonci A, Whistler JL (2005) Dopamine responsiveness is regulated by targeted sorting of D2 receptors. Proc Natl Acad Sci U S A 102:11521-11526.
Barton SC, Surani MAH, Norris ML (1984) Role of paternal and maternal genomes in mouse development. Nature 311:374-376.
Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley RA, Gluckman P, Godfrey K, Kirkwood T, Lahr MM (2004) Developmental plasticity and human health. Nature 430:419-421.
Bauer CT, Banks ML, Blough BE, Negus SS (2013) Use of intracranial self‐stimulation to evaluate abuse‐related and abuse‐limiting effects of monoamine releasers in rats. British journal of pharmacology 168:850-862.
Bauer M, Szulc J, Meyer M, Jensen CH, Terki TA, Meixner A, Kinkl N, Gasser T, Aebischer P, Ueffing M (2008) Delta‐like 1 participates in the specification of ventral midbrain progenitor derived dopaminergic neurons. J Neurochem 104:1101-1115.
Bayol SA, Farrington SJ, Stickland NC (2007) A maternal 'junk food'diet in pregnancy and lactation promotes an exacerbated taste for 'junk food'and a greater propensity for obesity in rat offspring. British Journal of Nutrition 98:843-851.
Beaulieu J-M, Gainetdinov RR (2011) The physiology, signaling, and pharmacology of dopamine receptors. Pharmacological reviews 63:182-217.
Becker JB (1999) Gender differences in dopaminergic function in striatum and nucleus accumbens. Pharmacology Biochemistry and Behavior 64:803-812.
Begemann M, Spengler S, Kanber D, Haake A, Baudis M, Leisten I, Binder G, Markus S, Rupprecht T, Segerer H (2011) Silver‐Russell patients showing a broad range of ICR1 and ICR2 hypomethylation in different tissues. Clin Genet 80:83-88.
Beiderbeck DI, Reber SO, Havasi A, Bredewold R, Veenema AH, Neumann ID (2012) High and abnormal forms of aggression in rats with extremes in trait anxiety–Involvement of the dopamine system in the nucleus accumbens. Psychoneuroendocrinology 37:1969-1980.
Bell AC, Felsenfeld G (2000) Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature 405:482-485.
Benloucif S, Galloway MP (1991) Facilitation of dopamine release in vivo by serotonin agonists: studies with microdialysis. European journal of pharmacology 200:1-8.
Berger MA, Barros VG, Sarchi MI, Tarazi FI, Antonelli MC (2002) Long-term effects of prenatal stress on dopamine and glutamate receptors in adult rat brain. Neurochemical research 27:1525-1533.

Bergman J, Roof RA, Furman CA, Conroy JL, Mello NK, Sibley DR, Skolnick P (2013) Modification of cocaine self-administration by buspirone (Buspar®): potential involvement of D3 and D4 dopamine receptors. The International Journal of Neuropsychopharmacology 16:445-458.

Berridge KC (1996) Food reward: brain substrates of wanting and liking. Neuroscience & Biobehavioral Reviews 20:1-25.

Berridge KC, Robinson TE, Aldridge JW (2009) Dissecting components of reward:'liking','wanting', and learning. Current opinion in pharmacology 9:65-73.

Bestor TH (1999) Sex brings transposons and genomes into conflict. Genetica 107:289-295.

Bestor TH (2003) Cytosine methylation mediates sexual conflict. Trends in Genetics 19:185-190.

Beyer C, Green SJ, Hutchison JB (1994a) Androgens influence sexual differentiation of embryonic mouse hypothalamic aromatase neurons in vitro. Endocrinology 135:1220-1226.

Beyer C, Green SJ, Barker PJ, Huskisson NS, Hutchinson JB (1994b) Aromatase-immunoreactivity is localised specifically in neurones in the developing mouse hypothalamus and cortex. Brain research 638:203-210.

Bhogal B, Arnaudo A, Dymkowski A, Best A, Davis TL (2004) Methylation at mouse *Cdkn1c* is acquired during postimplantation development and functions to maintain imprinted expression. Genomics 84:961-970.

Blissett J, Harris G, Kirk J (2001) Feeding problems in Silver‐Russell syndrome. Developmental Medicine & Child Neurology 43:39-44.

Bobzean SA, DeNobrega AK, Perrotti LI (2014) Sex differences in the neurobiology of drug addiction. Exp Neurol.

Boccaccio I, Glatt-Deeley H, Watrin F, Roëckel N, Lalande M, Muscatelli F (1999) The human MAGEL2 gene and its mouse homologue are paternally expressed and mapped to the Prader-Willi region. Hum Mol Genet 8:2497-2505.

Bonaldi A, Mazzeu JF, Costa SS, Honjo RS, Bertola DR, Albano LMJ, Furquim IM, Kim CA, Vianna‐Morgante AM (2011) Microduplication of the ICR2 domain at chromosome 11p15 and familial Silver–Russell syndrome. American Journal of Medical Genetics Part A 155:2479-2483.

Boughter JD, Jr., Baird JP, Bryant J, St John SJ, Heck D (2007) C57BL/6J and DBA/2J mice vary in lick rate and ingestive microstructure. Genes Brain Behav 6:619-627.

Bourc'his D, Xu G-L, Lin C-S, Bollman B, Bestor TH (2001) Dnmt3L and the establishment of maternal genomic imprints. Science 294:2536-2539.

Brandvain Y, Van Cleve J, Ubeda F, Wilkins JF (2011) Demography, kinship, and the evolving theory of genomic imprinting. Trends Genet 27:251-257.

Bridges RS, DiBiase R, Loundes DD, Doherty PC (1985) Prolactin stimulation of maternal behavior in female rats. Science 227:782-784.

Broad KD, Keverne EB (2011) Placental protection of the fetal brain during short-term food deprivation. Proc Natl Acad Sci U S A 108:15237-15241.

Broad KD, Curley JP, Keverne EB (2009) Increased apoptosis during neonatal brain development underlies the adult behavioral deficits seen in mice lacking a functional paternally expressed gene 3 (Peg3). Dev Neurobiol 69:314-325.

Brown AS, van Os J, Driessens C, Hoek HW, Susser ES (2000) Further evidence of relation between prenatal famine and major affective disorder. Am J Psychiatry 157:190-195.

Brown HD, McCutcheon JE, Cone JJ, Ragozzino ME, Roitman MF (2011) Primary food reward and reward‐predictive stimuli evoke different patterns of phasic dopamine signaling throughout the striatum. European Journal of Neuroscience 34:1997-2006.

Buckingham-Howes S, Berger SS, Scaletti LA, Black MM (2013) Systematic review of prenatal cocaine exposure and adolescent development. Pediatrics 131:e1917-1936.

Bullman H, Lever M, Robinson DO, Mackay DJG, Holder SE, Wakeling EL (2008) Mosaic maternal uniparental disomy of chromosome 11 in a patient with Silver–Russell syndrome. Journal of medical genetics 45:396-399.

Bunzow JR, Tol HHMV, Grandy DK, Albert P, Salon J, Christie M, Machida CA, Neve KA, Civelli O (1988) Cloning and expression of a rat D 2 dopamine receptor cDNA. Nature 336:783-787.

Cantone I, Fisher AG (2013) Epigenetic programming and reprogramming during development. Nat Struct Mol Biol 20:282-289.

Carlezon WA, Thome J, Olson VG, Lane-Ladd SB, Brodkin ES, Hiroi N, Duman RS, Neve RL, Nestler EJ (1998) Regulation of cocaine reward by CREB. Science 282:2272-2275.

Carlin J, George R, Reyes TM (2013) Methyl donor supplementation blocks the adverse effects of maternal high fat diet on offspring physiology. PLoS One 8:e63549.

Carmack SA, Howell KK, Rasaei K, Reas ET, Anagnostaras SG (2014) Animal model of methylphenidate's long-term memory-enhancing effects. Learning & Memory 21:82-89.

Carter RJ, Lione LA, Humby T, Mangiarini L, Mahal A, Bates GP, Dunnett SB, Morton AJ (1999) Characterization of progressive motor deficits in mice transgenic for the human Huntington's disease mutation. The Journal of neuroscience 19:3248-3257.

Caspary T, Cleary MA, Baker CC, Guan X-J, Tilghman SM (1998) Multiple mechanisms regulate imprinting of the mouse distal chromosome 7 gene cluster. Mol Cell Biol 18:3466-3474.

Caspary T, Cleary MA, Perlman EJ, Zhang P, Elledge SJ, Tilghman SM (1999) Oppositely imprinted genes p57Kip2 and Igf2 interact in a mouse model for Beckwith–Wiedemann syndrome. Genes Dev 13:3115-3124.

Castner SA, Xiao L, Becker JB (1993) Sex differences in striatal dopamine: in vivo microdialysis and behavioral studies. Brain research 610:127-134.

Cattanach BM, Kirk M (1985) Differential activity of maternally and paternally derived chromosome regions in mice. Nature 315:496-498.

Cattanach BM, Beechey CV, Peters J (2004) Interactions between imprinting effects in the mouse. Genetics 168:397-413.

Cerqueira A, Martín A, Symonds CE, Odajima J, Dubus P, Barbacid M, Santamaría D (2014) Genetic Characterization of the Role of the Cip/Kip Family of Proteins as Cyclin-Dependent Kinase Inhibitors and Assembly Factors. Mol Cell Biol 34:1452-1459.

Cerrato F, Sparago A, Di Matteo I, Zou X, Dean W, Sasaki H, Smith P, Genesio R, Bruggemann M, Reik W (2005) The two-domain hypothesis in Beckwith–

Wiedemann syndrome: autonomous imprinting of the telomeric domain of the distal chromosome 7 cluster. Hum Mol Genet 14:503-511.

Chai J-H, Locke DP, Ohta T, Greally JM, Nicholls RD (2001) Retrotransposed genes such as Frat3 in the mouse Chromosome 7C Prader-Willi syndrome region acquire the imprinted status of their insertion site. Mammalian Genome 12:813-821.

Chamberlain SJ, Johnstone KA, DuBose AJ, Simon TA, Bartolomei MS, Resnick JL, Brannan CI (2004) Evidence for genetic modifiers of postnatal lethality in PWS-IC deletion mice. Hum Mol Genet 13:2971-2977.

Champagne FA, Curley JP, Swaney WT, Hasen NS, Keverne EB (2009) Paternal influence on female behavior: the role of Peg3 in exploration, olfaction, and neuroendocrine regulation of maternal behavior of female mice. Behav Neurosci 123:469.

Charalambous M, Ward A, Hurst LD (2003a) Evidence for a priming effect on maternal resource allocation: implications for interbrood competition. Proceedings of the Royal Society of London Series B: Biological Sciences 270:S100-S103.

Charalambous M, da Rocha ST, Ferguson-Smith AC (2007) Genomic imprinting, growth control and the allocation of nutritional resources: consequences for postnatal life. Current Opinion in Endocrinology, Diabetes and Obesity 14:3-12.

Charalambous M, Smith FM, Bennett WR, Crew TE, Mackenzie F, Ward A (2003b) Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2-independent mechanism. Proceedings of the National Academy of Sciences 100:8292-8297.

Charalambous M, Cowley M, Geoghegan F, Smith FM, Radford EJ, Marlow BP, Graham CF, Hurst LD, Ward A (2010) Maternally-inherited *Grb10* reduces placental size and efficiency. Dev Biol 337:1-8.

Charalambous M, Ferron SR, da Rocha ST, Murray AJ, Rowland T, Ito M, Schuster-Gossler K, Hernandez A, Ferguson-Smith AC (2012) Imprinted gene dosage is critical for the transition to independent life. Cell Metab 15:209-221.

Chen DY, Stern SA, Garcia-Osta A, Saunier-Rebori B, Pollonini G, Bambah-Mukku D, Blitzer RD, Alberini CM (2011) A critical role for IGF-II in memory consolidation and enhancement. Nature 469:491-497.

Chen J, Kelz MB, Hope BT, Nakabeppu Y, Nestler EJ (1997) Chronic Fos-related antigens: stable variants of deltaFosB induced in brain by chronic treatments. J Neurosci 17:4933-4941.

Chiesa N, De Crescenzo A, Mishra K, Perone L, Carella M, Palumbo O, Mussa A, Sparago A, Cerrato F, Russo S, Lapi E, Cubellis MV, Kanduri C, Cirillo Silengo M, Riccio A, Ferrero GB (2012) The KCNQ1OT1 imprinting control region and non-coding RNA: new properties derived from the study of Beckwith-Wiedemann syndrome and Silver-Russell syndrome cases. Hum Mol Genet 21:10-25.

Choi JD, Underkoffler LA, Wood AJ, Collins JN, Williams PT, Golden JA, Schuster EF, Loomes KM, Oakey RJ (2005) A novel variant of Inpp5f is imprinted in brain, and its expression is correlated with differential methylation of an internal CpG island. Mol Cell Biol 25:5514-5522.

Christophersen NS, Grønborg M, Petersen TN, Fjord-Larsen L, Jørgensen JR, Juliusson B, Blom N, Rosenblad C, Brundin P (2007) Midbrain expression of Delta-like 1 homologue is regulated by GDNF and is associated with dopaminergic differentiation. Exp Neurol 204:791-801.

Ciccone DN, Su H, Hevi S, Gay F, Lei H, Bajko J, Xu G, Li E, Chen T (2009) KDM1B is a histone H3K4 demethylase required to establish maternal genomic imprints. Nature 461:415-418.

Claycombe KJ, Uthus EO, Roemmich JN, Johnson LK, Johnson WT (2013) Prenatal Low-Protein and Postnatal High-Fat Diets Induce Rapid Adipose Tissue Growth by Inducing Igf2 Expression in Sprague Dawley Rat Offspring. The Journal of nutrition 143:1533-1539.

Cohn DW, Gabanyi I, Kinoshita D, de Sa-Rocha LC (2012) Lipopolysaccharide administration in the dominant mouse destabilizes social hierarchy. Behav Processes 91:54-60.

Collier G, Novell K (1967) Saccharin as a sugar surrogate. Journal of comparative and physiological psychology 64:404.

Cordeiro A, Neto AP, Carvalho F, Ramalho C, Dória S (2014) Relevance of genomic imprinting in intrauterine human growth expression of CDKN1C, H19, IGF2, KCNQ1 and PHLDA2 imprinted genes. Journal of assisted reproduction and genetics:1-8.

Cordero MI, Sandi C (2007) Stress amplifies memory for social hierarchy. Frontiers in neuroscience 1:175.

Costa LM, Yuan J, Rouster J, Paul W, Dickinson H, Gutierrez-Marcos JF (2012) Maternal control of nutrient allocation in plant seeds by genomic imprinting. Curr Biol 22:160-165.

Covelo IR, Wirtshafter D, Stratford TR (2012) $GABA_A$ and dopamine receptors in the nucleus accumbens shell differentially influence performance of a water-reinforced progressive ratio task. Pharmacology Biochemistry and Behavior 101:57-61.

Cowley M, Garfield AS, Madon-Simon M, Charalambous M, Clarkson RW, Smalley MJ, Kendrick H, Isles AR, Parry AJ, Carney S, Oakey RJ, Heisler LK, Moorwood K, Wolf JB, Ward A (2014) Developmental programming mediated by complementary roles of imprinted grb10 in mother and pup. PLoS biology 12:e1001799.

Crawley JN (1999) Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. Brain research 835:18-26.

Cullen CL, Burne THJ, Lavidis NA, Moritz KM (2013) Low dose prenatal ethanol exposure induces anxiety-like behaviour and alters dendritic morphology in the basolateral amygdala of rat offspring. PLoS One 8:e54924.

Curley JP (2011) Is there a genomically imprinted social brain? Bioessays 33:662-668.

Curley JP, Mashoodh R (2010) Parent-of-origin and trans-generational germline influences on behavioral development: the interacting roles of mothers, fathers, and grandparents. Dev Psychobiol 52:312-330.

Curley JP, Barton S, Surani A, Keverne EB (2004) Coadaptation in mother and infant regulated by a paternally expressed imprinted gene. Proc Biol Sci 271:1303-1309.

da Rocha ST, Charalambous M, Lin SP, Gutteridge I, Ito Y, Gray D, Dean W, Ferguson-Smith AC (2009) Gene dosage effects of the imprinted delta-like homologue 1 (dlk1/pref1) in development: implications for the evolution of imprinting. PLoS Genet 5:e1000392.

Dahlstroem A, Fuxe K (1964) Evidence for the Existence of Monoamine-Containing Neurons in the Central Nervous System. I. Demonstration of Monoamines in the Cell Bodies of Brain Stem Neurons. Acta Physiol Scand Suppl:SUPPL 232:231-255.

Dal Toso R, Sommer B, Ewert M, Herb A, Pritchett DB, Bach A, Shivers BD, Seeburg PH (1989) The dopamine D2 receptor: two molecular forms generated by alternative splicing. The EMBO journal 8:4025.

Dalley JW, Theobald DE, Eagle DM, Passetti F, Robbins TW (2001) Deficits in impulse control associated with tonically-elevated serotonergic function in rat prefrontal cortex.

Darvas M, Palmiter RD (2009) Restriction of dopamine signaling to the dorsolateral striatum is sufficient for many cognitive behaviors. Proc Natl Acad Sci U S A 106:14664-14669.

Davies W, Smith RJ, Kelsey G, Wilkinson LS (2004) Expression patterns of the novel imprinted genes *Nap1l5* and *Peg13* and their non-imprinted host genes in the adult mouse brain. Gene expression patterns 4:741-747.

Davies W, Isles AR, Humby T, Wilkinson LS (2007) What are imprinted genes doing in the brain? Epigenetics 2:201-206.

Davis JD (1989) The microstructure of ingestive behavior. Annals of the New York Academy of Sciences 575:106-121.

Davis JD (1998) A model for the control of ingestion-20 years later. PROGRESS IN PSYCHOBIOLOGY AND PHYSIOLOGICAL PSYCHOLOGY, VOL 17 17:127-173.

Davis JD, Smith GP (1992) Analysis of the microstructure of the rhythmic tongue movements of rats ingesting maltose and sucrose solutions. Behav Neurosci 106:217.

Davis JD, Perez MC (1993) Food deprivation-and palatability-induced microstructural changes in ingestive behavior. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 264:R97-R103.

De Crescenzo A, Sparago A, Cerrato F, Palumbo O, Carella M, Miceli M, Bronshtein M, Riccio A, Yaron Y (2013) Paternal deletion of the 11p15.5 centromeric-imprinting control region is associated with alteration of imprinted gene expression and recurrent severe intrauterine growth restriction. Journal of medical genetics 50:99-103.

De Koning MB, Bloemen OJN, Van Duin EDA, Booij J, Abel KM, De Haan L, Linszen DH, Van Amelsvoort TAMJ (2014) Pre-pulse inhibition and striatal dopamine in subjects at an ultra-high risk for psychosis. Journal of Psychopharmacology 28:553-560.

de la Puente A, Hall J, Wu Y-Z, Leone G, Peters J, Yoon B-J, Soloway P, Plass C (2002) Structural characterization of *Rasgrf1* and a novel linked imprinted locus. Gene 291:287-297.

Dearry A, Gingrich JA, Falardeau P, Fremeau RT, Jr., Bates MD, Caron MG (1990) Molecular cloning and expression of the gene for a human D1 dopamine receptor. Nature 347:72-76.

DeLong MR (2000) The Basal Ganglia. In: Principles of Neural Science, 4th Edition (Kandel ER, Schwartz JH, Jessell TM, eds), pp 853-867. International Edition: McGraw-Hill.

Demars J, Gicquel C (2012) Epigenetic and genetic disturbance of the imprinted 11p15 region in Beckwith-Wiedemann and Silver-Russell syndromes. Clin Genet 81:350-361.

Demars J, Shmela ME, Khan AW, Lee KS, Azzi S, Dehais P, Netchine I, Rossignol S, Le Bouc Y, El-Osta A (2014) Genetic variants within the second intron of the KCNQ1 gene affect CTCF binding and confer a risk of Beckwith–Wiedemann syndrome upon maternal transmission. Journal of medical genetics 51:502-511.

Dennis K, Fan T, Geiman T, Yan Q, Muegge K (2001) Lsh, a member of the SNF2 family, is required for genome-wide methylation. Genes Dev 15:2940-2944.

Dent CL, Isles AR (2014) Brain-expressed imprinted genes and adult behaviour: the example of Nesp and Grb10. Mammalian Genome 25:87-93.

DeVeale B, Van Der Kooy D, Babak T (2012) Critical evaluation of imprinted gene expression by RNA–Seq: a new perspective. PLoS Genet 8:e1002600.

Devi YS, Halperin J (2014) Reproductive actions of prolactin mediated through short and long receptor isoforms. Mol Cell Endocrinol 382:400-410.

Dickinson A, Smith J, Mirenowicz J (2000) Dissociation of Pavlovian and instrumental incentive learning under dopamine antagonists. Behav Neurosci 114:468.

Docherty LE, Poole RL, Mattocks CJ, Lehmann A, Temple IK, Mackay DJG (2010) Further refinement of the critical minimal genetic region for the imprinting disorder 6q24 transient neonatal diabetes. Diabetologia 53:2347-2351.

Doe CM, Relkovic D, Garfield AS, Dalley JW, Theobald DE, Humby T, Wilkinson LS, Isles AR (2009) Loss of the imprinted snoRNA mbii-52 leads to increased 5htr2c pre-RNA editing and altered 5HT2CR-mediated behaviour. Hum Mol Genet 18:2140-2148.

Dotson CD, Spector AC (2005) Drinking spout orifice size affects licking behavior in inbred mice. Physiol Behav 85:655-661.

Drake AJ, Liu L, Kerrigan D, Meehan RR, Seckl JR (2011) Multigenerational programming in the glucocorticoid programmed rat is associated with generation-specific and parent of origin effects. Epigenetics 6:1334-1343.

Drew MR, Simpson EH, Kellendonk C, Herzberg WG, Lipatova O, Fairhurst S, Kandel ER, Malapani C, Balsam PD (2007) Transient overexpression of striatal D2 receptors impairs operant motivation and interval timing. The Journal of neuroscience 27:7731-7739.

Drews C (1993) The concept and definition of dominance in animal behaviour. Behaviour:283-313.

Drickamer LC (2001) Urine marking and social dominance in male house mice (< i> Mus musculus domesticus</i>). Behav Processes 53:113-120.

Duffié R, Ajjan S, Greenberg MV, Zamudio N, del Arenal ME, Iranzo J, Okamoto I, Barbaux S, Fauque P, Bourc'his D (2014) The Gpr1/Zdbf2 locus provides new paradigms for transient and dynamic genomic imprinting in mammals. Genes Dev 28:463-478.

Dumartin B, Jaber M, Gonon F, Caron MG, Giros B, Bloch B (2000) Dopamine tone regulates D1 receptor trafficking and delivery in striatal neurons in dopamine transporter-deficient mice. Proceedings of the National Academy of Sciences 97:1879-1884.

Düring S, Glenthøj BY, Andersen GS, Oranje B (2014) Effects of Dopamine D2/D3 Blockade on Human Sensory and Sensorimotor gating in Initially Antipsychotic-Naïve, First-Episode Schizophrenia Patients. Neuropsychopharmacology.

Dwyer DM, Lydall ES, Hayward AJ (2011) Simultaneous contrast: Evidence from licking microstructure and cross-solution comparisons. Journal of Experimental Psychology: Animal Behavior Processes 37:200.

Dwyer DM, Pincham HL, Thein T, Harris JA (2009) A learned flavor preference persists despite the extinction of conditioned hedonic reactions to the cue flavors. Learn Behav 37:305-310.

Dyer MA, Cepko CL (2000) p57 (Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina. Development 127:3593-3605.

Ebbesen P, Villadsen JA, Villadsen HD, Heller KE (1992) Effect of social order, lack of social hierarchy, and restricted feeding on murine survival and virus leukemia. Annals of the New York Academy of Sciences 673:46-52.

Edwards DH, Kravitz EA (1997) Serotonin, social status and aggression. Current opinion in neurobiology 7:812-819.

Eggermann T, Begemann M, Binder G, Spengler S (2010) Silver-Russell syndrome: genetic basis and molecular genetic testing. Orphanet J Rare Dis 5:19.

Engemann S, Strödicke M, Paulsen M, Franck O, Reinhardt R, Lane N, Reik W, Walter J (2000) Sequence and functional comparison in the Beckwith–Wiedemann region: implications for a novel imprinting centre and extended imprinting. Hum Mol Genet 9:2691-2706.

Enklaar T, Zabel BU, Prawitt D (2006) Beckwith-Wiedemann syndrome: multiple molecular mechanisms. Expert Rev Mol Med 8:1-19.

Entringer S (2013) Impact of stress and stress physiology during pregnancy on child metabolic function and obesity risk. Current opinion in clinical nutrition and metabolic care 16:320.

Erickson JD, Eiden LE, Hoffman BJ (1992) Expression cloning of a reserpine-sensitive vesicular monoamine transporter. Proceedings of the National Academy of Sciences 89:10993-10997.

Esel E, Basturk M, Saffet Gonul A, Kula M, Tayfun Turan M, Yabanoglu I, Sofuoglu S (2001) Effects of olanzapine and haloperidol on serum prolactin levels in male schizophrenic patients. Psychoneuroendocrinology 26:641-647.

Evans HK, Weidman JR, Cowley DO, Jirtle RL (2005) Comparative phylogenetic analysis of Blcap/Nnat reveals eutherian-specific imprinted gene. Molecular Biology and Evolution 22:1740-1748.

Fan T, Hagan JP, Kozlov SV, Stewart CL, Muegge K (2005) Lsh controls silencing of the imprinted Cdkn1c gene. Development 132:635-644.

Faure A, Richard JM, Berridge KC (2010) Desire and dread from the nucleus accumbens: cortical glutamate and subcortical GABA differentially generate motivation and hedonic impact in the rat. PLoS One 5:e11223.

Ferguson-Smith AC (2011) Genomic imprinting: the emergence of an epigenetic paradigm. Nature Reviews Genetics 12:565-575.
Ferguson-Smith AC, Cattanach BM, Barton SC, Beechey CV, Surani MA (1991) Embryological and molecular investigations of parental imprinting on mouse chromosome 7. Nature 351:667-670.
Ferron SR, Charalambous M, Radford E, McEwen K, Wildner H, Hind E, Morante-Redolat JM, Laborda J, Guillemot F, Bauer SR, Farinas I, Ferguson-Smith AC (2011) Postnatal loss of Dlk1 imprinting in stem cells and niche astrocytes regulates neurogenesis. Nature 475:381-385.
Figueroa-Guzmán Y, Reilly S (2008) NMDA receptors in the basolateral amygdala and gustatory neophobia. Brain research 1210:200-203.
File SE (2001) Factors controlling measures of anxiety and responses to novelty in the mouse. Behav Brain Res 125:151-157.
Finger BC, Dinan TG, Cryan JF (2010) Progressive ratio responding in an obese mouse model: Effects of fenfluramine. Neuropharmacology 59:619-626.
Fitzpatrick GV, Soloway PD, Higgins MJ (2002) Regional loss of imprinting and growth deficiency in mice with a targeted deletion of KvDMR1. Nat Genet 32:426-431.
Fitzpatrick GV, Pugacheva EM, Shin J-Y, Abdullaev Z, Yang Y, Khatod K, Lobanenkov VV, Higgins MJ (2007) Allele-specific binding of CTCF to the multipartite imprinting control region KvDMR1. Mol Cell Biol 27:2636-2647.
Flores G, de Jesús Gómez-Villalobos M, Rodríguez-Sosa L (2011) Prenatal Amphetamine Exposure Effects on Dopaminergic Receptors and Transporter in Postnatal Rats. Neurochemical research 36:1740-1749.
Frank D, Fortino W, Clark L, Musalo R, Wang W, Saxena A, Li C-M, Reik W, Ludwig T, Tycko B (2002) Placental overgrowth in mice lacking the imprinted gene Ipl. Proceedings of the National Academy of Sciences 99:7490-7495.
Franzek EJ, Sprangers N, Janssens A, Van Duijn CM, Van De Wetering BJM (2008) Prenatal exposure to the 1944–45 Dutch 'hunger winter'and addiction later in life. Addiction 103:433-438.
Freed WJ, Chen J, Backman CM, Schwartz CM, Vazin T, Cai J, Spivak CE, Lupica CR, Rao MS, Zeng X (2008) Gene expression profile of neuronal progenitor cells derived from hESCs: activation of chromosome 11p15.5 and comparison to human dopaminergic neurons. PLoS One 3:e1422.
Fride E, Dan Y, Feldon J, Halevy G, Weinstock M (1986) Effects of prenatal stress on vulnerability to stress in prepubertal and adult rats. Physiol Behav 37:681-687.
Frost JM, Moore GE (2010) The importance of imprinting in the human placenta. PLoS Genet 6:e1001015.
Frost WN, Tian L-M, Hoppe TA, Mongeluzi DL, Wang J (2003) A cellular mechanism for prepulse inhibition. Neuron 40:991-1001.
Fujiwara K, Hasegawa K, Ohkumo T, Miyoshi H, Tseng Y-H, Yoshikawa K (2012) Necdin controls proliferation of white adipocyte progenitor cells. PLoS One 7:e30948.
Furutachi S, Matsumoto A, Nakayama KI, Gotoh Y (2013) p57 controls adult neural stem cell quiescence and modulates the pace of lifelong neurogenesis. The EMBO journal 32:970-981.

Gadow KD, Pinsonneault JK, Perlman G, Sadee W (2014) Association of dopamine gene variants, emotion dysregulation and ADHD in autism spectrum disorder. Research in developmental disabilities 35:1658-1665.

Gainetdinov RR (1999) Role of Serotonin in the Paradoxical Calming Effect of Psychostimulants on Hyperactivity. Science 283:397-401.

Galler JR, Tonkiss J (1991) Prenatal protein malnutrition and maternal behavior in Sprague-Dawley rats. The Journal of nutrition 121:762-769.

Gallou-Kabani C, Gabory A, Tost J, Karimi M, Mayeur S, Lesage J, Boudadi E, Gross MS, Taurelle J, Vige A, Breton C, Reusens B, Remacle C, Vieau D, Ekstrom TJ, Jais JP, Junien C (2010) Sex- and diet-specific changes of imprinted gene expression and DNA methylation in mouse placenta under a high-fat diet. PLoS One 5:e14398.

Garfield AS, Cowley M, Smith FM, Moorwood K, Stewart-Cox JE, Gilroy K, Baker S, Xia J, Dalley JW, Hurst LD, Wilkinson LS, Isles AR, Ward A (2011) Distinct physiological and behavioural functions for parental alleles of imprinted Grb10. Nature 469:534-538.

Gburcik V, Cleasby ME, Timmons JA (2013) Loss of neuronatin promotes "browning" of primary mouse adipocytes while reducing Glut1-mediated glucose disposal. American Journal of Physiology-Endocrinology and Metabolism 304:E885-E894.

Gerfen CR, Engber TM, Mahan LC, Susel Z, Chase TN, Monsma FJ, Jr., Sibley DR (1990) D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. Science 250:1429-1432.

Gicquel C, Rossignol S, Cabrol S, Houang M, Steunou V, Barbu V, Danton F, Thibaud N, Le Merrer M, Burglen L (2005) Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver-Russell syndrome. Nat Genet 37:1003-1007.

Giros B, Jaber M, Jones SR, Wightman RM, Caron MG (1996) Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature 379:15.

Godoy MAd, Souza ASd, Lobo MA, Sampaio OVK, Moraes L, Baldanza MR, Magri TPR, Wernerck de Castro JPS, Tavares do Carmo MdG, Soares-Mota M (2013) Effects of protein restriction during gestation and lactation on cell proliferation in the hippocampus and subventricular zone: Functional implications. Protein restriction alters hippocampal/SVZ cell proliferation. Brain research 1496:10-27.

Golden SA, Covington HE, 3rd, Berton O, Russo SJ (2011) A standardized protocol for repeated social defeat stress in mice. Nat Protoc 6:1183-1191.

Gong L, Pan Y-X, Chen H (2010) Gestational low protein diet in the rat mediatesIgf2gene expression in male offspring via altered hepatic DNA methylation. Epigenetics 5:619-626.

Gould TD, Pfeifer K (1998) Imprinting of mouse Kvlqt1 is developmentally regulated. Hum Mol Genet 7:483-487.

Graham FK (1975) The more or less startling effects of weak prestimulation. Psychophysiology 12:238-248.

Grandjean V, Smith J, Schofield PN, Ferguson-Smith AC (2000) Increased IGF-II protein affects p57kip2 expression in vivo and in vitro: implications for

Beckwith–Wiedemann syndrome. Proceedings of the National Academy of Sciences 97:5279-5284.
Grandy DK, Zhang YA, Bouvier C, Zhou QY, Johnson RA, Allen L, Buck K, Bunzow JR, Salon J, Civelli O (1991) Multiple human D5 dopamine receptor genes: a functional receptor and two pseudogenes. Proc Natl Acad Sci U S A 88:9175-9179.
Gray S, Hurst JL (1995) The effects of cage cleaning on aggression within groups of male laboratory mice. Animal behaviour 49:821-826.
Graybiel AM, Moratalla R, Robertson HA (1990) Amphetamine and cocaine induce drug-specific activation of the c-fos gene in striosome-matrix compartments and limbic subdivisions of the striatum. Proceedings of the National Academy of Sciences 87:6912-6916.
Gregg C, Zhang J, Weissbourd B, Luo S, Schroth GP, Haig D, Dulac C (2010) High-resolution analysis of parent-of-origin allelic expression in the mouse brain. Science 329:643-648.
Gressens P, Muaku SM, Besse L, Nsegbe E, Gallego J, Delpech B, Gaultier C, Evrard P, Ketelslegers J-M, Maiter D (1997) Maternal protein restriction early in rat pregnancy alters brain development in the progeny. Developmental brain research 103:21-35.
Grissom NM, Lyde R, Christ L, Sasson IE, Carlin J, Vitins AP, Simmons RA, Reyes TM (2013) Obesity at conception programs the opioid system in the offspring brain. Neuropsychopharmacology.
Groenewegen HJ (2003) The basal ganglia and motor control. Neural plasticity 10:107-120.
Groves PM, Boyle RD, Welker RL, Miller SW (1974) On the mechanism of prepulse inhibition. Physiol Behav 12:367-375.
Gui H, Li S, Matise MP (2007) A cell-autonomous requirement for Cip/Kip cyclin-kinase inhibitors in regulating neuronal cell cycle exit but not differentiation in the developing spinal cord. Dev Biol 301:14-26.
Guibert S, Forné T, Weber M (2012) Global profiling of DNA methylation erasure in mouse primordial germ cells. Genome research 22:633-641.
Guitart X, Thompson MA, Mirante CK, Greenberg ME, Nestler EJ (1992) Regulation of cyclic AMP response element‐binding protein (CREB) phosphorylation by acute and chronic morphine in the rat locus coeruleus. J Neurochem 58:1168-1171.
Guo L, Choufani S, Ferreira J, Smith A, Chitayat D, Shuman C, Uxa R, Keating S, Kingdom J, Weksberg R (2008) Altered gene expression and methylation of the human chromosome 11 imprinted region in small for gestational age (SGA) placentae. Dev Biol 320:79-91.
Haber SN, Ryoo H, Cox C, Lu W (1995) Subsets of midbrain dopaminergic neurons in monkeys are distinguished by different levels of mRNA for the dopamine transporter: comparison with the mRNA for the D2 receptor, tyrosine hydroxylase and calbindin immunoreactivity. J Comp Neurol 362:400-410.
Haig D (1994) Refusing the ovarian time bomb: three viewpoints and a reply. Trends in Genetics 10:346-347.
Haig D (2000) The kinship theory of genomic imprinting. Annual review of ecology and systematics 31:9-32.

Haig D (2004) Genomic imprinting and kinship: how good is the evidence? Annu Rev Genet 38:553-585.
Haig D (2012) Retroviruses and the placenta. Current Biology 22:R609-R613.
Haig D (2013) Coadaptation and conflict, misconception and muddle, in the evolution of genomic imprinting. Heredity.
Haig D (2014) Troubled sleep Night waking, breastfeeding and parent–offspring conflict. Evolution, medicine, and public health 2014:32-39.
Haig D, Wilkins JF (2000) Genomic imprinting, sibling solidarity and the logic of collective action. Philosophical Transactions of the Royal Society of London Series B: Biological Sciences 355:1593-1597.
Haig D, Úbeda F (2011) Genomic imprinting: an obsession with depilatory mice. Current Biology 21:R257-R259.
Hajkova P, Erhardt S, Lane N, Haaf T, El-Maarri O, Reik W, Walter J, Surani MA (2002) Epigenetic reprogramming in mouse primordial germ cells. Mechanisms of development 117:15-23.
Hajkova P, Ancelin K, Waldmann T, Lacoste N, Lange UC, Cesari F, Lee C, Almouzni G, Schneider R, Surani MA (2008) Chromatin dynamics during epigenetic reprogramming in the mouse germ line. Nature 452:877-881.
Hajnal A, Smith GP, Norgren R (2004) Oral sucrose stimulation increases accumbens dopamine in the rat. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 286:R31-R37.
Hall JG (1990) Genomic imprinting: review and relevance to human diseases. Am J Hum Genet 46:857.
Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM (2000) CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature 405:486-489.
Hatada I, Mukai T (1995) Genomic imprinting of p57KIP2, a cyclin–dependent kinase inhibitor, in mouse. Nat Genet 11:204-206.
Hatada I, Ohashi H, Fukushima Y, Kaneko Y, Inoue M, Komoto Y, Okada A, Ohishi S, Nabetani A, Morisaki H, Nakayama M, Niikawa N, Mukai T (1996a) An imprinted gene p57KIP2 is mutated in Beckwith-Wiedemann syndrome. Nat Genet 14:171-173.
Hatada I, Inazawa J, Abe T, Nakayama M, Kaneko Y, Jinno Y, Niikawa N, Ohashi H, Fukushima Y, Iida K, Yutani C, Takahashi S, Chiba Y, Ohishi S, Mukai T (1996b) Genomic imprinting of human p57KIP2 and its reduced expression in Wilms' tumors. Hum Mol Genet 5:783-788.
Hausknecht K, Haj-Dahmane S, Shen RY (2013) Prenatal stress exposure increases the excitation of dopamine neurons in the ventral tegmental area and alters their reponses to psychostimulants. Neuropsychopharmacology 38:293-301.
Havecker ER, Gao X, Voytas DF (2004) The diversity of LTR retrotransposons. GENOME BIOLOGY 5:225-225.
Hernandez L, Hoebel BG (1988a) Food reward and cocaine increase extracellular dopamine in the nucleus accumbens as measured by microdialysis. Life sciences 42:1705-1712.
Hernandez L, Hoebel BG (1988b) Feeding and hypothalamic stimulation increase dopamine turnover in the accumbens. Physiol Behav 44:599-606.

Hernandez L, Kozlov S, Piras G, Stewart CL (2003) Paternal and maternal genomes confer opposite effects on proliferation, cell-cycle length, senescence, and tumor formation. Proceedings of the National Academy of Sciences 100:13344-13349.

Hess EJ, Albers LJ, Le H, Creese I (1986) Effects of chronic SCH23390 treatment on the biochemical and behavioral properties of D1 and D2 dopamine receptors: potentiated behavioral responses to a D2 dopamine agonist after selective D1 dopamine receptor upregulation. Journal of Pharmacology and Experimental Therapeutics 238:846-854.

Hikichi T, Kohda T, Kaneko‐Ishino T, Ishino F (2003) Imprinting regulation of the murine Meg1/Grb10 and human GRB10 genes; roles of brain‐specific promoters and mouse‐specific CTCF‐binding sites. Nucleic acids research 31:1398-1406.

Hirasawa R, Chiba H, Kaneda M, Tajima S, Li E, Jaenisch R, Sasaki H (2008) Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. Genes Dev 22:1607-1616.

Holman L, Kokko H (2013) The evolution of genomic imprinting: costs, benefits and long‐term consequences. Biological Reviews doi: 10.1111/brv.12069.

Hope BT, Nye HE, Kelz MB, Self DW, Iadarola MJ, Nakabeppu Y, Duman RS, Nestler EJ (1994) Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. Neuron 13:1235-1244.

Hurley MJ, Jenner P (2006) What has been learnt from study of dopamine receptors in Parkinson's disease? Pharmacology & therapeutics 111:715-728.

Hurst JL (1993) The priming effects of urine substrate marks on interactions between male house mice, Mus musculus domesticus Schwarz & Schwarz. Animal behaviour.

Hurst JL, Fang J, Barnard CJ (1993) The role of substrate odours in maintaining social tolerance between male house mice, *Mus musculus domesticus*. Animal behaviour 45:997-1006.

Hurst LD (1997) 12 l Evolutionary theories of genomic imprinting. Genomic imprinting:211.

Hutchison KE, Swift R (1999) Effect of d-amphetamine on prepulse inhibition of the startle reflex in humans. Psychopharmacology (Berl) 143:394-400.

Hutter B, Helms V, Paulsen M (2006) Tandem repeats in the CpG islands of imprinted genes. Genomics 88:323-332.

Huttunen MO, Niskanen P (1978) Prenatal loss of father and psychiatric disorders. Archives of general psychiatry 35:429.

Ishino Y, Hayashi Y, Naruse M, Tomita K, Sanbo M, Fuchigami T, Fujiki R, Hirose K, Toyooka Y, Fujimori T (2014) Bre1a, a Histone H2B Ubiquitin Ligase, Regulates the Cell Cycle and Differentiation of Neural Precursor Cells. The Journal of neuroscience 34:3067-3078.

Isles AR, Wilkinson LS (2000) Imprinted genes, cognition and behaviour. Trends Cogn Sci 4:309-318.

Isles AR, Davies W, Wilkinson LS (2006) Genomic imprinting and the social brain. Philos Trans R Soc Lond B Biol Sci 361:2229-2237.

Itoh Y, Masuyama N, Nakayama K, Nakayama KI, Gotoh Y (2007) The cyclin-dependent kinase inhibitors p57 and p27 regulate neuronal migration in the developing mouse neocortex. J Biol Chem 282:390-396.

Ivanova E, Chen J-H, Segonds-Pichon A, Ozanne SE, Kelsey G (2012) DNA methylation at differentially methylated regions of imprinted genes is resistant to developmental programming by maternal nutrition. Epigenetics 7:1200-1210.

Izvolskaia M, Duittoz AH, Tillet Y, Ugrumov MV (2009) The influence of catecholamine on the migration of gonadotropin-releasing hormone-producing neurons in the rat foetuses. Brain Structure and Function 213:289-300.

Jacobs FMJ, Van der Linden AJA, Wang Y, von Oerthel L, Sul HS, Burbach JPH, Smidt MP (2009) Identification of Dlk1, Ptpru and Klhl1 as novel Nurr1 target genes in meso-diencephalic dopamine neurons. Development 136:2363-2373.

Jacobson JL, Jacobson SW (2002) Effects of prenatal alcohol exposure on child development. Alcohol Res Health 26:282-286.

Jadasz JJ, Rivera FJ, Taubert A, Kandasamy M, Sandner B, Weidner N, Aktas O, Hartung HP, Aigner L, Kury P (2012) p57kip2 regulates glial fate decision in adult neural stem cells. Development 139:3306-3315.

Jahng JW, Houpt TA, Wessel TC, Chen K, Shih JC, Joh TH (1997) Localization of monoamine oxidase A and B mRNA in the rat brain by in situ hybridization. Synapse 25:30-36.

Jemiolo B, Xie T-M, Novotny M (1992) Urine marking in male mice: responses to natural and synthetic chemosignals. Physiol Behav 52:521-526.

Jensen CH, Meyer M, Schroder HD, Kliem A, Zimmer J, Teisner B (2001) Neurons in the monoaminergic nuclei of the rat and human central nervous system express FA1/dlk. Neuroreport 12:3959-3963.

Jensen Peña C, Neugut YD, Calarco CA, Champagne FA (2014) Effects of maternal care on the development of midbrain dopamine pathways and reward‐directed behavior in female offspring. European Journal of Neuroscience 39:946-956.

Jiang Y-h, Pan Y, Zhu L, Landa L, Yoo J, Spencer C, Lorenzo I, Brilliant M, Noebels J, Beaudet AL (2010) Altered ultrasonic vocalization and impaired learning and memory in Angelman syndrome mouse model with a large maternal deletion from Ube3a to Gabrb3. PLoS One 5:e12278.

Joaquin M, Gubern A, González‐Nuñez D, Josue Ruiz E, Ferreiro I, de Nadal E, Nebreda AR, Posas F (2012) The p57 CDKi integrates stress signals into cell‐cycle progression to promote cell survival upon stress. The EMBO journal 31:2952-2964.

John RM, Lefebvre L (2011) Developmental regulation of somatic imprints. Differentiation 81:270-280.

John RM, Ainscough JFX, Barton SC, Surani MA (2001) Distant cis-elements regulate imprinted expression of the mouse p57 Kip2 (Cdkn1c) gene: implications for the human disorder, Beckwith–Wiedemann syndrome. Hum Mol Genet 10:1601-1609.

John RM, Hodges M, Little P, Barton SC, Surani MA (1999) A human p57KIP2 transgene is not activated by passage through the maternal mouse germline. Hum Mol Genet 8:2211-2219.

Johnson AW, Sherwood A, Smith DR, Wosiski-Kuhn M, Gallagher M, Holland PC (2010a) An analysis of licking microstructure in three strains of mice. Appetite 54:320-330.

Johnson ML, Ho CC, Day AE, Walker QD, Francis R, Kuhn CM (2010b) Oestrogen receptors enhance dopamine neurone survival in rat midbrain. J Neuroendocrinol 22:226-237.

Jones KL, Smith RM, Edwards KS, Givens B, Tilley MR, Beversdorf DQ (2010) Combined effect of maternal serotonin transporter genotype and prenatal stress in modulating offspring social interaction in mice. Int J Dev Neurosci 28:529-536.

Jones S, Kornblum JL, Kauer JA (2000) Amphetamine blocks long-term synaptic depression in the ventral tegmental area. The Journal of neuroscience 20:5575-5580.

Jones SR, Gainetdinov RR, Wightman RM, Caron MG (1998) Mechanisms of amphetamine action revealed in mice lacking the dopamine transporter. The Journal of neuroscience 18:1979-1986.

Joseph B, Wallen-Mackenzie A, Benoit G, Murata T, Joodmardi E, Okret S, Perlmann T (2003) p57(Kip2) cooperates with Nurr1 in developing dopamine cells. Proc Natl Acad Sci U S A 100:15619-15624.

Joseph B, Andersson ER, Vlachos P, Sodersten E, Liu L, Teixeira AI, Hermanson O (2009) p57Kip2 is a repressor of Mash1 activity and neuronal differentiation in neural stem cells. Cell Death Differ 16:1256-1265.

Joseph MH, Hodges H (1990) Lever pressing for food reward and changes in dopamine turnover and uric acid in rat caudate and nucleus accumbens studied chronically by in vivo voltammetry. Journal of neuroscience methods 34:143-149.

Kanber D, Buiting K, Roos C, Gromoll J, Kaya S, Horsthemke B, Lohmann D (2013) The Origin of the RB1 Imprint. PLoS One 8:e81502.

Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, Li E, Sasaki H (2004) Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature 429:900-903.

Kannenberg K, Urban C, Binder G (2012) Increased incidence of aberrant DNA methylation within diverse imprinted gene loci outside of IGF2/H19 in Silver-Russell syndrome. Clin Genet 81:366-377.

Kanno K, Kokubo H, Takahashi A, Koide T, Ishiura S (2014) Enhanced prepulse inhibition and low sensitivity to a dopamine agonist in HESR1 knockout mice. Journal of neuroscience research 92:287-297.

Kant SG, Van der Weij AM, Oostdijk W, Wit JM, Robinson DO, Temple IK, Mackay DJG (2005) Monozygous triplets discordant for transient neonatal diabetes mellitus and for imprinting of the TNDM differentially methylated region. Human genetics 117:398-401.

Kareta MS, Botello ZM, Ennis JJ, Chou C, Chédin F (2006) Reconstitution and mechanism of the stimulation of de novo methylation by human DNMT3L. Journal of Biological Chemistry 281:25893-25902.

Kato Y, Kaneda M, Hata K, Kumaki K, Hisano M, Kohara Y, Okano M, Li E, Nozaki M, Sasaki H (2007) Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse. Hum Mol Genet 16:2272-2280.

Kaufman MH, Barton SC, Surani MAH (1977) Normal postimplantation development of mouse parthenogenetic embryos to the forelimb bud stage. Nature 265:53-55.
Kelsey G, Feil R (2013) New insights into establishment and maintenance of DNA methylation imprints in mammals. Philosophical Transactions of the Royal Society B: Biological Sciences 368:20110336.
Kelz MB, Chen J, Carlezon WA, Jr., Whisler K, Gilden L, Beckmann AM, Steffen C, Zhang YJ, Marotti L, Self DW, Tkatch T, Baranauskas G, Surmeier DJ, Neve RL, Duman RS, Picciotto MR, Nestler EJ (1999) Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. Nature 401:272-276.
Keshavan MS, Tandon R, Boutros NN, Nasrallah HA (2008) Schizophrenia,"just the facts": What we know in 2008: Part 3: Neurobiology. Schizophrenia research 106:89-107.
Keshet GI, Weinstock M (1995) Maternal naltrexone prevents morphological and behavioral alterations induced in rats by prenatal stress. Pharmacol Biochem Behav 50:413-419.
Keverne EB (2012) Significance of epigenetics for understanding brain development, brain evolution and behaviour. Neuroscience.
Keverne EB, Fundele R, Narasimha M, Barton SC, Surani MA (1996) Genomic imprinting and the differential roles of parental genomes in brain development. Developmental brain research 92:91-100.
Kim J, Frey WD, He H, Kim H, Ekram MB, Bakshi A, Faisal M, Perera BPU, Ye A, Teruyama R (2013a) Peg3 Mutational Effects on Reproduction and Placenta-Specific Gene Families. PLoS One 8:e83359.
Kim P, Park JH, Choi CS, Choi I, Joo SH, Kim MK, Kim SY, Kim KC, Park SH, Kwon KJ (2013b) Effects of ethanol exposure during early pregnancy in hyperactive, inattentive and impulsive behaviors and MeCP2 expression in rodent offspring. Neurochemical research 38:620-631.
Kim SW, Fowler JS, Skolnick P, Muench L, Kang Y, Shea C, Logan J, Kim D, Carter P, King P (2014) Therapeutic doses of buspirone block D3 receptors in the living primate brain. The International Journal of Neuropsychopharmacology:1-11.
King V, Hibbert N, Seckl JR, Norman JE, Drake AJ (2013) The effects of an obesogenic diet during pregnancy on fetal growth and placental gene expression are gestation dependent. Placenta 34:1087-1090.
Kirlic N, Newman E, LaGasse LL, Derauf C, Shah R, Smith LM, Arria AM, Huestis MA, Haning W, Strauss A (2013) Cortisol Reactivity in Two-Year-Old Children Prenatally Exposed to Methamphetamine. Journal of studies on alcohol and drugs 74:447.
Kobayashi H, Sakurai T, Imai M, Takahashi N, Fukuda A, Yayoi O, Sato S, Nakabayashi K, Hata K, Sotomaru Y (2012) Contribution of intragenic DNA methylation in mouse gametic DNA methylomes to establish oocyte-specific heritable marks. PLoS Genet 8:e1002440.
Kobayashi H, Yanagisawa E, Sakashita A, Sugawara N, Kumakura S, Ogawa H, Akutsu H, Hata K, Nakabayashi K, Kono T (2013) Epigenetic and transcriptional features of the novel human imprinted lncRNA GPR1AS suggest it is a functional ortholog to mouse Zdbf2linc. Epigenetics 8:635-645.

Kobayashi S, Kohda T, Ichikawa H, Ogura A, Ohki M, Kaneko-Ishino T, Ishino F (2002) Paternal Expression of a Novel Imprinted Gene,< i> Peg12/Frat3,</i> in the Mouse 7C Region Homologous to the Prader–Willi Syndrome Region. Biochemical and biophysical research communications 290:403-408.

Kono T, Obata Y, Wu Q, Niwa K, Ono Y, Yamamoto Y, Park ES, Seo J-S, Ogawa H (2004) Birth of parthenogenetic mice that can develop to adulthood. Nature 428:860-864.

Kotzot D, Schmitt S, Bernasconi F, Robinson WP, Lurie IW, Ilyina H, Méhes K, Hamel BCJ, Otten BJ, Hergersberg M (1995) Uniparental disomy 7 in Silver—Russell syndrome and primordial growth retardation. Hum Mol Genet 4:583-587.

Krakowski M (2003) Violence and serotonin: influence of impulse control, affect regulation, and social functioning. The Journal of neuropsychiatry and clinical neurosciences 15:294-305.

Kritzer MF, Creutz LM (2008) Region and sex differences in constituent dopamine neurons and immunoreactivity for intracellular estrogen and androgen receptors in mesocortical projections in rats. The Journal of neuroscience 28:9525-9535.

Kwong WY, Miller DJ, Ursell E, Wild AE, Wilkins AP, Osmond C, Anthony FW, Fleming TP (2006) Imprinted gene expression in the rat embryo–fetal axis is altered in response to periconceptional maternal low protein diet. Reproduction 132:265-277.

Laborie LB, Mackay DJG, Temple IK, Molven A, Søvik O, Njølstad PR (2010) DNA hypomethylation, transient neonatal diabetes, and prune belly sequence in one of two identical twins. European journal of pediatrics 169:207-213.

Laplante DP, Barr RG, Brunet A, Du Fort GG, Meaney ML, Saucier J-F, Zelazo PR, King S (2004) Stress during pregnancy affects general intellectual and language functioning in human toddlers. Pediatric Research 56:400-410.

Latos PA, Pauler FM, Koerner MV, Senergin HB, Hudson QJ, Stocsits RR, Allhoff W, Stricker SH, Klement RM, Warczok KE, Aumayr K, Pasierbek P, Barlow DP (2012) Airn transcriptional overlap, but not its lncRNA products, induces imprinted Igf2r silencing. Science 338(6113):1469-72.

Lee M-H, Reynisdottir I, Massague J (1995) Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev 9:639-649.

Lee MP, DeBaun MR, Mitsuya K, Galonek HL, Brandenburg S, Oshimura M, Feinberg AP (1999) Loss of imprinting of a paternally expressed transcript, with antisense orientation to KVLQT1, occurs frequently in Beckwith–Wiedemann syndrome and is independent of insulin-like growth factor II imprinting. Proceedings of the National Academy of Sciences 96:5203-5208.

Lefebvre L, Viville S, Barton SC, Ishino F, Keverne EB, Surani MA (1998) Abnormal maternal behaviour and growth retardation associated with loss of the imprinted gene Mest. Nat Genet 20:163-169.

Lewis A, Mitsuya K, Constancia M, Reik W (2004) Tandem repeat hypothesis in imprinting: deletion of a conserved direct repeat element upstream of

H19 has no effect on imprinting in the Igf2-H19 region. Mol Cell Biol 24:5650-5656.
Lex A, Hauber W (2008) Dopamine D1 and D2 receptors in the nucleus accumbens core and shell mediate Pavlovian-instrumental transfer. Learning & Memory 15:483-491.
Li E, Beard C, Jaenisch R (1993) Role for DNA methylation in genomic imprinting. Nature 366:362-365.
Li LL, Keverne EB, Aparicio SA, Ishino F, Barton SC, Surani MA (1999) Regulation of maternal behavior and offspring growth by paternally expressed Peg3. Science 284:330-334.
Li X, Ito M, Zhou F, Youngson N, Zuo X, Leder P, Ferguson-Smith AC (2008) A Maternal-Zygotic Effect Gene,*Zfp57*, Maintains Both Maternal and Paternal Imprints. Developmental cell 15:547-557.
Lieb K, Andersen C, Lazarov N, Zienecker R, Urban I, Reisert I, Pilgrim C (1996) Pre-and postnatal development of dopaminergic neuron numbers in the male and female mouse midbrain. Developmental brain research 94:37-43.
Lin S-P, Youngson N, Takada S, Seitz H, Reik W, Paulsen M, Cavaille J, Ferguson-Smith AC (2003) Asymmetric regulation of imprinting on the maternal and paternal chromosomes at the Dlk1-Gtl2 imprinted cluster on mouse chromosome 12. Nat Genet 35:97-102.
Lin SP, Coan P, da Rocha ST, Seitz H, Cavaille J, Teng PW, Takada S, Ferguson-Smith AC (2007) Differential regulation of imprinting in the murine embryo and placenta by the Dlk1-Dio3 imprinting control region. Development 134:417-426.
Lin XB, Pierce DR, Light KE, Hayar A (2013) The Fine Temporal Structure of the Rat Licking Pattern: What Causes the Variabiliy in the Interlick Intervals and How is it Affected by the Drinking Solution? Chemical senses 38:685-704.
Lin Y, Zhuo Y, Fang Z-f, Che L-q, Wu D (2012) Effect of maternal dietary energy types on placenta nutrient transporter gene expressions and intrauterine fetal growth in rats. Nutrition 28:1037-1043.
Lindzey G, Winston H, Manosevitz M (1961) Social dominance in inbred mouse strains. Nature 194:474-476.
Lister RG (1987) The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 92:180-185.
Lister RG (1990) Ethologically-based animal models of anxiety disorders. Pharmacology & therapeutics 46:321-340.
Lo H-W (2013) Akt destabilizes p57Kip2: Akt at the converging crossroad? Cell Cycle 12:870.
Loiseau F, Millan MJ (2009) Blockade of dopamine D_3 receptors in frontal cortex, but not in sub-cortical structures, enhances social recognition in rats: Similar actions of D_1 receptor agonists, but not of D_2 antagonists. European Neuropsychopharmacology 19:23-33.
Long SY (1972) Hair-nibbling and whisker-trimming as indicators of social hierarchy in mice. Animal behaviour 20:10-12.
Lucion A, Vogel WH (1994) Effects of stress on defensive aggression and dominance in a water competition test. Integrative Physiological and Behavioral Science 29:415-422.

Lydall ES, Gilmour G, Dwyer DM (2010) Analysis of licking microstructure provides no evidence for a reduction in reward value following acute or sub-chronic phencyclidine administration. Psychopharmacology (Berl) 209:153-162.

Lyons DJ, Broberger C (2014) TIDAL WAVES: Network mechanisms in the neuroendocrine control of prolactin release. Frontiers in Neuroendocrinology.

MacLeod RM, Lehmeyer JE (1974) Studies on the mechanism of the dopamine-mediated inhibition of prolactin secretion. Endocrinology 94:1077-1085.

Madhavan A, Argilli E, Bonci A, Whistler JL (2013) Loss of D2 dopamine receptor function modulates cocaine-induced glutamatergic synaptic potentiation in the ventral tegmental area. The Journal of neuroscience 33:12329-12336.

Mahler SV, Smith KS, Berridge KC (2007) Endocannabinoid hedonic hotspot for sensory pleasure: anandamide in nucleus accumbens shell enhances 'liking'of a sweet reward. Neuropsychopharmacology 32:2267-2278.

Malaspina D, Corcoran C, Kleinhaus KR, Perrin MC, Fennig S, Nahon D, Friedlander Y, Harlap S (2008) Acute maternal stress in pregnancy and schizophrenia in offspring: a cohort prospective study. BMC psychiatry 8:71.

Mancini-DiNardo D (2003) A differentially methylated region within the gene Kcnq1 functions as an imprinted promoter and silencer. Hum Mol Genet 12:283-294.

Mancini-Dinardo D, Steele SJ, Levorse JM, Ingram RS, Tilghman SM (2006) Elongation of the Kcnq1ot1 transcript is required for genomic imprinting of neighboring genes. Genes Dev 20:1268-1282.

Mann M, Varmuza S (1994) Refusing the ovarian time bomb: three viewpoints and a reply. Trends in Genetics 10:348-349.

Manuck SB, Flory JD, Ferrell RE, Mann JJ, Muldoon MF (2000) A regulatory polymorphism of the monoamine oxidase-A gene may be associated with variability in aggression, impulsivity, and central nervous system serotonergic responsivity. Psychiatry Res 95:9-23.

Manuel-Apolinar L, Zarate A, Rocha L, Hernández M (2010) Fetal malnutrition affects hypothalamic leptin receptor expression after birth in male mice. Archives of medical research 41:240-245.

Manuel‐Apolinar L, Rocha L, Damasio L, Tesoro‐Cruz E, Zarate A (2014) Role of prenatal undernutrition in the expression of serotonin, dopamine and leptin receptors in adult mice: Implications of food intake. Molecular medicine reports 9:407-412.

Matsumoto M, Yoshioka M, Togashi H, Ikeda T, Saito H (1996) Functional regulation by dopamine receptors of serotonin release from the rat hippocampus: in vivo microdialysis study. Naunyn Schmiedebergs Arch Pharmacol 353:621-629.

Matsumoto M, Togashi H, Mori K, Ueno K, Miyamoto A, Yoshioka M (1999) Characterization of endogenous serotonin-mediated regulation of dopamine release in the rat prefrontal cortex. European journal of pharmacology 383:39-48.

Matsuoka S, Edwards MC, Bai C, Parker S, Zhang P, Baldini A, Harper JW, Elledge SJ (1995) p57KIP2, a structurally distinct member of the p21CIP1 Cdk

inhibitor family, is a candidate tumor suppressor gene. Genes Dev 9:650-662.
Matsuoka S, Thompson JS, Edwards MC, Bartletta JM, Grundy P, Kalikin LM, Harper JW, Elledge SJ, Feinberg AP (1996) Imprinting of the gene encoding a human cyclin-dependent kinase inhibitor, p57KIP2, on chromosome 11p15. Proc Natl Acad Sci U S A 93:3026-3030.
Mattson SN, Calarco KE, Lang AR (2006) Focused and shifting attention in children with heavy prenatal alcohol exposure. Neuropsychology 20:361-369.
McArthur S, McHale E, Gillies GE (2006) The size and distribution of midbrain dopaminergic populations are permanently altered by perinatal glucocorticoid exposure in a sex-region-and time-specific manner. Neuropsychopharmacology 32:1462-1476.
McDonald JF, Matzke MA, Matzke AJ (2005) Host defenses to transposable elements and the evolution of genomic imprinting. Cytogenetic and genome research 110:242-249.
McGrath J, Solter D (1984a) Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell 37:179-183.
McGrath J, Solter D (1984b) Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell 1984 May;37(1):179-83.
McLaughlin KJ, Szabó P, Haegel H, Mann JR (1996) Mouse embryos with paternal duplication of an imprinted chromosome 7 region die at midgestation and lack placental spongiotrophoblast. Development 122:265-270.
McMinn J, Wei M, Schupf N, Cusmai J, Johnson EB, Smith AC, Weksberg R, Thaker HM, Tycko B (2006) Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. Placenta 27:540-549.
McNamara GI, Isles AR (2014) Influencing the Social Group: The Role of Imprinted Genes. Advances in Genetics 86:107-134.
Meijer A (1985) Child psychiatric sequelae of maternal war stress. Acta Psychiatrica Scandinavica 72:505-511.
Migeon BR, Wolf SF, Axelman J, Kaslow DC, Schmidt M (1985) Incomplete X chromosome dosage compensation in chorionic villi of human placenta. Proceedings of the National Academy of Sciences 82:3390-3394.
Mikaelsson MA, Constância M, Dent CL, Wilkinson LS, Humby T (2013) Placental programming of anxiety in adulthood revealed by Igf2-null models. Nature communications 4.
Miller GM (2011) The emerging role of trace amine‐associated receptor 1 in the functional regulation of monoamine transporters and dopaminergic activity. J Neurochem 116:164-176.
Miyoshi N, Kuroiwa Y, Kohda T, Shitara H, Yonekawa H, Kawabe T, Hasegawa H, Barton SC, Surani MA, Kaneko-Ishino T (1998) Identification of the Meg1/Grb10 imprinted gene on mouse proximal chromosome 11, a candidate for the Silver–Russell syndrome gene. Proceedings of the National Academy of Sciences 95:1102-1107.
Mizoguchi H, Yamada K, Mouri A, Niwa M, Mizuno T, Noda Y, Nitta A, Itohara S, Banno Y, Nabeshima T (2007) Role of matrix metalloproteinase and tissue inhibitor of MMP in methamphetamine‐induced behavioral

sensitization and reward: implications for dopamine receptor down‐regulation and dopamine release. J Neurochem 102:1548-1560.
Mohammad F, Mondal T, Guseva N, Pandey GK, Kanduri C (2010) Kcnq1ot1 noncoding RNA mediates transcriptional gene silencing by interacting with Dnmt1. Development 137:2493-2499.
Moles A, Bartolomucci A, Garbugino L, Conti R, Caprioli A, Coccurello R, Rizzi R, Ciani B, D'Amato FR (2006) Psychosocial stress affects energy balance in mice: modulation by social status. Psychoneuroendocrinology 31:623-633.
Moore T (1994) Refusing the ovarian time bomb: three viewpoints and a reply. Trends in Genetics 10:347-348.
Moore T, Haig D (1991) Genomic imprinting in mammalian development: a parental tug-of-war. Trends in Genetics 7:45-49.
Moratalla R, Elibol B, Vallejo M, Graybiel AM (1996) Network-level changes in expression of inducible Fos-Jun proteins in the striatum during chronic cocaine treatment and withdrawal. Neuron 17:147-156.
Morgan HD, Santos F, Green K, Dean W, Reik W (2005) Epigenetic reprogramming in mammals. Hum Mol Genet 14:R47-R58.
Morgane PJ, Austin-LaFrance R, Bronzino J, Tonkiss J, Diaz-Cintra S, Cintra L, Kemper T, Galler JR (1993) Prenatal malnutrition and development of the brain. Neuroscience & Biobehavioral Reviews 17:91-128.
Mount R, Oliver C, Berg K, Horsler K (2011) Effects of adult familiarity on social behaviours in Angelman syndrome. Journal of Intellectual Disability Research 55:339-350.
Naef L, Moquin L, Dal Bo G, Giros B, Gratton A, Walker CD (2011) Maternal high-fat intake alters presynaptic regulation of dopamine in the nucleus accumbens and increases motivation for fat rewards in the offspring. Neuroscience 176:225-236.
Nagano T, Mitchell JA, Sanz LA, Pauler FM, Ferguson-Smith AC, Feil R, Fraser P (2008) The Air noncoding RNA epigenetically silences transcription by targeting G9a to chromatin. Science 322:1717-1720.
Nakamura T, Arai Y, Umehara H, Masuhara M, Kimura T, Taniguchi H, Sekimoto T, Ikawa M, Yoneda Y, Okabe M (2007) PGC7/Stella protects against DNA demethylation in early embryogenesis. Nature Cell Biology 9:64-71.
Nakamura T, Liu Y-J, Nakashima H, Umehara H, Inoue K, Matoba S, Tachibana M, Ogura A, Shinkai Y, Nakano T (2012) PGC7 binds histone H3K9me2 to protect against conversion of 5mC to 5hmC in early embryos. Nature 486:415-419.
Napolitano F, Bonito-Oliva A, Federici M, Carta M, Errico F, Magara S, Martella G, Nistico R, Centonze D, Pisani A, Gu HH, Mercuri NB, Usiello A (2010) Role of aberrant striatal dopamine D1 receptor/cAMP/protein kinase A/DARPP32 signaling in the paradoxical calming effect of amphetamine. J Neurosci 30:11043-11056.
Nelson AC, Colson KE, Harmon S, Potts WK (2013) Rapid adaptation to mammalian sociality via sexually selected traits. BMC Evol Biol 13:81.
Nestler EJ (2001) Molecular basis of long-term plasticity underlying addiction. Nature Reviews Neuroscience 2:119-128.
Nestler EJ, Carlezon Jr WA (2006) The mesolimbic dopamine reward circuit in depression. Biol Psychiatry 59:1151-1159.

Nestler EJ, Barrot M, Self DW (2001) DeltaFosB: a sustained molecular switch for addiction. Proc Natl Acad Sci U S A 98:11042-11046.

Nguyen L, Besson A, Heng JI-T, Schuurmans C, Teboul L, Parras C, Philpott A, Roberts JM, Guillemot F (2006) p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. Genes Dev 20:1511-1524.

Nicholls RD, Knepper JL (2001) Genome organization, function, and imprinting in Prader-Willi and Angelman syndromes. Annu Rev Genomics Hum Genet 2:153-175.

Niemitz EL, DeBaun MR, Fallon J, Murakami K, Kugoh H, Oshimura M, Feinberg AP (2004) Microdeletion of LIT1 in familial Beckwith-Wiedemann syndrome. Am J Hum Genet 75:844-849.

Noain D, Perez-Millan MI, Bello EP, Luque GM, Casas Cordero R, Gelman DM, Peper M, Tornadu IG, Low MJ, Becu-Villalobos D, Rubinstein M (2013) Central dopamine D2 receptors regulate growth-hormone-dependent body growth and pheromone signaling to conspecific males. J Neurosci 33:5834-5842.

O'Neill B, Gu HH (2013) Amphetamine-induced locomotion in a hyperdopaminergic ADHD mouse model depends on genetic background. Pharmacol Biochem Behav 103:455-459.

Ohlsson R, Hedborg F, Holmgren L, Walsh C, Ekstrom TJ (1994) Overlapping patterns of IGF2 and H19 expression during human development: biallelic IGF2 expression correlates with a lack of H19 expression. Development 120:361-368.

Okae H, Hiura H, Nishida Y, Funayama R, Tanaka S, Chiba H, Yaegashi N, Nakayama K, Sasaki H, Arima T (2011) Re-investigation and RNA sequencing-based identification of genes with placenta-specific imprinted expression. Hum Mol Genet:ddr488.

Oliver C, Horsler K, Berg K, Bellamy G, Dick K, Griffiths E (2007) Genomic imprinting and the expression of affect in Angelman syndrome: what's in the smile? Journal of Child Psychology and Psychiatry 48:571-579.

Ostlund SB, Maidment NT (2012) Dopamine receptor blockade attenuates the general incentive motivational effects of noncontingently delivered rewards and reward-paired cues without affecting their ability to bias action selection. Neuropsychopharmacology 37:508-519.

Ouchi Y, Banno Y, Shimizu Y, Ando S, Hasegawa H, Adachi K, Iwamoto T (2013) Reduced adult hippocampal neurogenesis and working memory deficits in the Dgcr8-deficient mouse model of 22q11. 2 deletion-associated schizophrenia can be rescued by IGF2. The Journal of neuroscience 33:9408-9419.

Palmer AA, Brown AS, Keegan D, Siska LD, Susser E, Rotrosen J, Butler PD (2008) Prenatal protein deprivation alters dopamine-mediated behaviors and dopaminergic and glutamatergic receptor binding. Brain research 1237:62-74.

Pang X, Hogan EM, Casserly A, Gao G, Gardner PD, Tapper AR (2014) Dicer expression is essential for adult midbrain dopaminergic neuron maintenance and survival. Molecular and Cellular Neuroscience 58:22-28.

Pask AJ, Papenfuss AT, Ager EI, McColl KA, Speed TP, Renfree MB (2009) Analysis of the platypus genome suggests a transposon origin for mammalian imprinting. Genome biology 10:R1.

Patten MM, Ross L, Curley JP, Queller DC, Bonduriansky R, Wolf JB (2014) The evolution of genomic imprinting: theories, predictions and empirical tests. Heredity.

Paulsen M, Davies KR, Bowden LM, Villar AJ, Franck O, Fuermann M, Dean WL, Moore TF, Rodrigues N, Davies KE (1998) Syntenic organization of the mouse distal chromosome 7 imprinting cluster and the Beckwith-Wiedemann syndrome region in chromosome 11p15. 5. Hum Mol Genet 7:1149-1159.

Paumier KL, Rizzo SJS, Berger Z, Chen Y, Gonzales C, Kaftan E, Li L, Lotarski S, Monaghan M, Shen W (2013) Behavioral Characterization of A53T Mice Reveals Early and Late Stage Deficits Related to Parkinson's Disease. PLoS One 8:e70274.

Pecina S, Cagniard B, Berridge KC, Aldridge JW, Zhuang X (2003) Hyperdopaminergic mutant mice have higher "wanting" but not "liking" for sweet rewards. The Journal of neuroscience 23:9395-9402.

Peciña S, Berridge KC (2013) Dopamine or opioid stimulation of nucleus accumbens similarly amplify cue‐triggered 'wanting' for reward: entire core and medial shell mapped as substrates for PIT enhancement. European Journal of Neuroscience 37:1529-1540.

Pedroza-Llinas R, Ramirez-Lugo L, Guzman-Ramos K, Zavala-Vega S, Bermudez-Rattoni F (2009) Safe taste memory consolidation is disrupted by a protein synthesis inhibitor in the nucleus accumbens shell. Neurobiology of learning and memory 92:45-52.

Peñaherrera MS, Weindler S, Van Allen MI, Yong SL, Metzger DL, McGillivray B, Boerkoel C, Langlois S, Robinson WP (2010) Methylation profiling in individuals with Russell–Silver syndrome. American Journal of Medical Genetics Part A 152:347-355.

Perry JRB*ea* (2014) Parent-of-origin-specific allelic associations among 106 genomic loci for age at menarche. Nature advance online publication.

Pham NV, Nguyen MT, Hu J-F, Vu TH, Hoffman AR (1998) Dissociation of *IGF2* and *H19* imprinting in human brain. Brain research 810:1-8.

Pielock SM, Lex B, Hauber W (2011) The role of dopamine in the dorsomedial striatum in general and outcome‐selective Pavlovian‐instrumental transfer. European Journal of Neuroscience 33:717-725.

Pittoggi C, Renzi L, Zaccagnini G, Cimini D, Degrassi F, Giordano R, Magnano AR, Lorenzini R, Lavia P, Spadafora C (1999) A fraction of mouse sperm chromatin is organized in nucleosomal hypersensitive domains enriched in retroposon DNA. Journal of cell science 112:3537-3548.

Plagge A, Gordon E, Dean W, Boiani R, Cinti S, Peters J, Kelsey G (2004) The imprinted signaling protein XLαs is required for postnatal adaptation to feeding. Nat Genet 36:818-826.

Plagge A, Isles AR, Gordon E, Humby T, Dean W, Gritsch S, Fischer-Colbrie R, Wilkinson LS, Kelsey G (2005) Imprinted Nesp55 influences behavioral reactivity to novel environments. Mol Cell Biol 25:3019-3026.

Poon K, Mandava S, Chen K, Barson JR, Buschlen S, Leibowitz SF (2013) Prenatal Exposure to Dietary Fat Induces Changes in the Transcriptional Factors,

TEF and YAP, Which May Stimulate Differentiation of Peptide Neurons in Rat Hypothalamus. PLoS One 8:e77668.
Popp C, Dean W, Feng S, Cokus SJ, Andrews S, Pellegrini M, Jacobsen SE, Reik W (2010) Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. Nature 463:1101-1105.
Prut L, Belzung C (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. European journal of pharmacology 463:3-33.
Radford EJ, Ferron SR, Ferguson-Smith AC (2011) Genomic imprinting as an adaptative model of developmental plasticity. FEBS Lett 585:2059-2066.
Radford EJ, Ito M, Shi H, Corish JA, Yamazawa K, Isganaitis E, Seisenberger S, Hore TA, Reik W, Erkek S (2014) In utero undernourishment perturbs the adult sperm methylome and intergenerational metabolism. Science 345(6198): 1255903.
Radford EJ, Isganaitis E, Jimenez-Chillaron J, Schroeder J, Molla M, Andrews S, Didier N, Charalambous M, McEwen K, Marazzi G, Sassoon D, Patti ME, Ferguson-Smith AC (2012) An unbiased assessment of the role of imprinted genes in an intergenerational model of developmental programming. PLoS Genet 8:e1002605.
Raleigh MJ, McGuire MT, Brammer GL, Pollack DB, Yuwiler A (1991) Serotonergic mechanisms promote dominance acquisition in adult male vervet monkeys. Brain research 559:181-190.
Ralph RJ, Paulus MP, Fumagalli F, Caron MG, Geyer MA (2001) Prepulse inhibition deficits and perseverative motor patterns in dopamine transporter knock-out mice: differential effects of D1 and D2 receptor antagonists. The Journal of neuroscience 21:305-313.
Ramos A (2008) Animal models of anxiety: do I need multiple tests? Trends in pharmacological sciences 29:493-498.
Rathbun W, Druse MJ (1985) Dopamine, Serotonin, and Acid Metabolites in Brain Regions from the Developing Offspring of Ethanol‐Treated Rats. J Neurochem 44:57-62.
Redgrave P, Rodriguez M, Smith Y, Rodriguez-Oroz MC, Lehericy S, Bergman H, Agid Y, DeLong MR, Obeso JA (2010) Goal-directed and habitual control in the basal ganglia: implications for Parkinson's disease. Nat Rev Neurosci 11:760-772.
Regier PS, Carroll ME, Meisel RL (2012) Cocaine-induced c-Fos expression in rats selectively bred for high or low saccharin intake and in rats selected for high or low impulsivity. Behav Brain Res 233:271-279.
Reik W, Dean W, Walter J (2001) Epigenetic reprogramming in mammalian development. Science 293:1089-1093.
Reisert I, Schuster R, Zienecker R, Pilgrim C (1990) Prenatal development of mesencephalic and diencephalic dopaminergic systems in the male and female rat. Developmental brain research 53:222-229.
Relkovic D, Doe CM, Humby T, Johnstone KA, Resnick JL, Holland AJ, Hagan JJ, Wilkinson LS, Isles AR (2010) Behavioural and cognitive abnormalities in an imprinting centre deletion mouse model for Prader-Willi syndrome. Eur J Neurosci 31:156-164.

Renfree MB, Suzuki S, Kaneko-Ishino T (2013) The origin and evolution of genomic imprinting and viviparity in mammals. Philosophical Transactions of the Royal Society B: Biological Sciences 368:20120151.

Reyes-Castro LA, Rodriguez JS, Charco R, Bautista CJ, Larrea F, Nathanielsz PW, Zambrano E (2012a) Maternal protein restriction in the rat during pregnancy and/or lactation alters cognitive and anxiety behaviors of female offspring. International Journal of Developmental Neuroscience 30:39-45.

Reyes-Castro LA, Rodriguez JS, Rodriguez-Gonzalez GL, Wimmer RD, McDonald TJ, Larrea F, Nathanielsz PW, Zambrano E (2011) Pre-and/or postnatal protein restriction in rats impairs learning and motivation in male offspring. International Journal of Developmental Neuroscience 29:177-182.

Reyes-Castro LA, Rodriguez JS, Rodriguez-Gonzalez GL, Chavira R, Bautista CJ, McDonald TJ, Nathanielsz PW, Zambrano E (2012b) Pre-and/or postnatal protein restriction developmentally programs affect and risk assessment behaviors in adult male rats. Behav Brain Res 227:324-329.

Rhees RW, Shryne JE, Gorski RA (1990) Onset of the hormone‐sensitive perinatal period for sexual differentiation of the sexually dimorphic nucleus of the preoptic area in female rats. J Neurobiol 21:781-786.

Rice F, Jones I, Thapar A (2007) The impact of gestational stress and prenatal growth on emotional problems in offspring: a review. Acta Psychiatrica Scandinavica 115:171-183.

Richards M, Hardy R, Kuh D, Wadsworth MEJ (2001) Birth weight and cognitive function in the British 1946 birth cohort: longitudinal population based study. British Medical Journal 322:199-203.

Robinson S, Rainwater AJ, Hnasko TS, Palmiter RD (2007) Viral restoration of dopamine signaling to the dorsal striatum restores instrumental conditioning to dopamine-deficient mice. Psychopharmacology (Berl) 191:567-578.

Robinson TE, Berridge KC (1993) The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain research reviews 18:247-291.

Robinson TE, Berridge KC (2008) The incentive sensitization theory of addiction: some current issues. Philosophical Transactions of the Royal Society B: Biological Sciences 363:3137-3146.

Rogner UC, Spyropoulos DD, Le Novère N, Changeux J-P, Avner P (2000) Control of neurulation by the nucleosome assembly protein-1–like 2. Nat Genet 25:431-435.

Romieu P, Host L, Gobaille S, Sandner G, Aunis D, Zwiller J (2008) Histone deacetylase inhibitors decrease cocaine but not sucrose self-administration in rats. The Journal of neuroscience 28:9342-9348.

Roussotte FF, Bramen JE, Nunez SC, Quandt LC, Smith L, O'Connor MJ, Bookheimer SY, Sowell ER (2011) Abnormal brain activation during working memory in children with prenatal exposure to drugs of abuse: the effects of methamphetamine, alcohol, and polydrug exposure. Neuroimage 54:3067-3075.

Sa-Rocha VM, Sa-Rocha LC, Palermo-Neto J (2006) Variations in behavior, innate immunity and host resistance to B16F10 melanoma growth in mice that present social stable hierarchical ranks. Physiol Behav 88:108-115.

Salamone JD, Wisniecki A, Carlson BB, Correa M (2001) Nucleus accumbens dopamine depletions make animals highly sensitive to high fixed ratio requirements but do not impair primary food reinforcement. Neuroscience 105:863-870.

Sanchez Vega MC, Chong S, Burne TH (2013) Early gestational exposure to moderate concentrations of ethanol alters adult behaviour in C57BL/6J mice. Behav Brain Res 252:326-333.

Sandman CA, Davis EP, Buss C, Glynn LM (2011) Exposure to prenatal psychobiological stress exerts programming influences on the mother and her fetus. Neuroendocrinology 95:8-21.

Sandner G, Canal NM (2007) Relationship between PPI and baseline startle response. Cognitive neurodynamics 1:27-37.

Santos F, Peters AH, Otte AP, Reik W, Dean W (2005) Dynamic chromatin modifications characterise the first cell cycle in mouse embryos. Dev Biol 280:225-236.

Sapolsky RM (2005) The influence of social hierarchy on primate health. Science 308:648-652.

Sari Y, Hammad LA, Saleh MM, Rebec GV, Mechref Y (2010) Alteration of selective neurotransmitters in fetal brains of prenatally alcohol-treated C57BL/6 mice: quantitative analysis using liquid chromatography/tandem mass spectrometry. International Journal of Developmental Neuroscience 28:263-269.

Sarna JR, Dyck RH, Whishaw IQ (2000) The Dalila effect: C57BL6 mice barber whiskers by plucking. Behav Brain Res 108:39-45.

Saunders BT, Robinson TE (2012) The role of dopamine in the accumbens core in the expression of Pavlovian‐conditioned responses. European Journal of Neuroscience 36:2521-2532.

Sauve D, Woodside B (1996) The effect of central administration of prolactin on food intake in virgin female rats is dose-dependent, occurs in the absence of ovarian hormones and the latency to onset varies with feeding regimen. Brain research 729:75-81.

Schmeisser MJ, Baumann B, Johannsen S, Vindedal GF, Jensen V, Hvalby ØC, Sprengel R, Seither J, Maqbool A, Magnutzki A (2012) IκB kinase/nuclear factor κB-dependent insulin-like growth factor 2 (Igf2) expression regulates synapse formation and spine maturation via Igf2 receptor signaling. The Journal of neuroscience 32:5688-5703.

Schmidt-Edelkraut U, Daniel G, Hoffmann A, Spengler D (2014) Zac1 regulates cell cycle arrest in neuronal progenitors via Tcf4. Mol Cell Biol 34:1020-1030.

Schmidt‐Edelkraut U, Hoffmann A, Daniel G, Spengler D (2013) Zac1 regulates astroglial differentiation of neural stem cells through socs3. Stem Cells 31:1621-1632.

Schönherr N, Meyer E, Roos A, Schmidt A, Wollmann HA, Eggermann T (2007) The centromeric 11p15 imprinting centre is also involved in Silver–Russell syndrome. Journal of medical genetics 44:59-63.

Schwartz JH (2000) Neurotransmitters. In: Principles of Neural Science, 4th Edition (Kandel ER, Schwartz JH, Jessell TM, eds), pp 280-297. International Edition: McGraw-Hill.

Schwarzkopf SB, Lamberti JS, Smith DA (1993) Concurrent assessment of acoustic startle and auditory P50 evoked potential measures of sensory inhibition. Biol Psychiatry 33:815-828.

Segovia KN, Correa M, Lennington JB, Conover JC, Salamone JD (2012) Changes in nucleus accumbens and neostriatal c‐Fos and DARPP‐32 immunoreactivity during different stages of food‐reinforced instrumental training. European Journal of Neuroscience 35:1354-1367.

Sesack SR, Aoki C, Pickel VM (1994) Ultrastructural localization of D2 receptor-like immunoreactivity in midbrain dopamine neurons and their striatal targets. J Neurosci 14:88-106.

Sferruzzi-Perri AN, Vaughan OR, Haro M, Cooper WN, Musial B, Charalambous M, Pestana D, Ayyar S, Ferguson-Smith AC, Burton GJ (2013) An obesogenic diet during mouse pregnancy modifies maternal nutrient partitioning and the fetal growth trajectory. The FASEB Journal 27:3928-3937.

Shin AC, Townsend RL, Patterson LM, Berthoud H-R (2011) "Liking" and "wanting" of sweet and oily food stimuli as affected by high-fat diet-induced obesity, weight loss, leptin, and genetic predisposition. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 301:R1267-R1280.

Shin J-Y, Fitzpatrick GV, Higgins MJ (2007) Two distinct mechanisms of silencing by the KvDMR1 imprinting control region. The EMBO journal 27:168-178.

Shingo T, Gregg C, Enwere E, Fujikawa H, Hassam R, Geary C, Cross JC, Weiss S (2003) Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. Science 299:117-120.

Siciliano CA, Calipari ES, Ferris MJ, Jones SR (2014) Biphasic Mechanisms of Amphetamine Action at the Dopamine Terminal. The Journal of neuroscience 34:5575-5582.

Skibicka KP, Shirazi RH, Rabasa-Papio C, Alvarez-Crespo M, Neuber C, Vogel H, Dickson SL (2013) Divergent circuitry underlying food reward and intake effects of ghrelin: dopaminergic VTA-accumbens projection mediates ghrelin's effect on food reward but not food intake. Neuropharmacology 73:274-283.

Sleutels F, Zwart R, Barlow DP (2002) The non-coding Air RNA is required for silencing autosomal imprinted genes. Nature 415:810-813.

Sloboda DM, Howie GJ, Pleasants A, Gluckman PD, Vickers MH (2009) Pre-and postnatal nutritional histories influence reproductive maturation and ovarian function in the rat. PLoS One 4:e6744.

Slotkin TA, Lau C, McCook EC, Lappi SE, Seidler FJ (1994) Glucocorticoids enhance intracellular signaling via adenylate cyclase at three distinct loci in the fetus: a mechanism for heterologous teratogenic sensitization? Toxicology and applied pharmacology 127:64-75.

Smidt MP, Burbach JPH (2007) How to make a mesodiencephalic dopaminergic neuron. Nature Reviews Neuroscience 8:21-32.

Smith FM, Garfield AS, Ward A (2006) Regulation of growth and metabolism by imprinted genes. Cytogenetic and genome research 113:279-291.

Smith JC, Sclafani A (2002) Saccharin as a sugar surrogate revisited. Appetite 38:155-160.

Sokoloff P, Giros B, Martres MP, Bouthenet ML, Schwartz JC (1990) Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. Nature 347:146-151.
Soloff PH, Price JC, Mason NS, Becker C, Meltzer CC (2010) Gender, personality, and serotonin-2A receptor binding in healthy subjects. Psychiatry Research: Neuroimaging 181:77-84.
Solter D (1988) Differential imprinting and expression of maternal and paternal genomes. Annu Rev Genet 22:127-146.
Solter D (1994) Refusing the ovarian time bomb: three viewpoints and a reply. Trends in Genetics 10:346.
Sonntag KC, Brenhouse HC, Freund N, Thompson BS, Puhl M, Andersen SL (2014) Viral over-expression of D1 dopamine receptors in the prefrontal cortex increase high-risk behaviors in adults: Comparison with adolescents. Psychopharmacology (Berl):1-12.
Sousa N, Almeida OFX, Wotjak CT (2006) A hitchhiker's guide to behavioral analysis in laboratory rodents. Genes, Brain and Behavior 5:5-24.
Sparago A, Cerrato F, Vernucci M, Ferrero GB, Silengo MC, Riccio A (2004) Microdeletions in the human H19 DMR result in loss of IGF2 imprinting and Beckwith-Wiedemann syndrome. Nat Genet 36:958-960.
Spector AC, Klumpp PA, Kaplan JM (1998) Analytical issues in the evaluation of food deprivation and sucrose concentration effects on the microstructure of licking behavior in the rat. Behav Neurosci 112:678.
St Clair D, Xu M, Wang P, Yu Y, Fang Y, Zhang F, Zheng X, Gu N, Feng G, Sham P (2005) Rates of adult schizophrenia following prenatal exposure to the Chinese famine of 1959-1961. Jama 294:557-562.
Stone JM, Davis JM, Leucht S, Pilowsky LS (2009) Cortical dopamine D2/D3 receptors are a common site of action for antipsychotic drugs—an original patient data meta-analysis of the SPECT and PET in vivo receptor imaging literature. Schizophrenia bulletin 35:789-797.
Strozik E, Festing MF (1981) Whisker trimming in mice. Lab Anim 15:309-312.
Sunahara RK, Guan HC, O'Dowd BF, Seeman P, Laurier LG, Ng G, George SR, Torchia J, Van Tol HH, Niznik HB (1991) Cloning of the gene for a human dopamine D5 receptor with higher affinity for dopamine than D1. Nature 350:614-619.
Surani MA, Barton SC (1983) Development of gynogenetic eggs in the mouse: implications for parthenogenetic embryos. Science 222:1034-1036.
Surani MAH, Barton SC, Norris ML (1984) Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature 308:548-550.
Surani MAH, Barton SC, Norris ML (1986) Nuclear transplantation in the mouse: heritable differences between parental genomes after activation of the embryonic genome. Cell 45:127-136.
Susiarjo M, Sasson I, Mesaros C, Bartolomei MS (2013) Bisphenol a exposure disrupts genomic imprinting in the mouse. PLoS Genet 9:e1003401.
Suzuki S, Shaw G, Kaneko-Ishino T, Ishino F, Renfree MB (2011a) The evolution of mammalian genomic imprinting was accompanied by the acquisition of novel CpG islands. Genome biology and evolution 3:1276-1283.

Suzuki S, Shaw G, Kaneko-Ishino T, Ishino F, Renfree MB (2011b) Characterisation of marsupial PHLDA2 reveals eutherian specific acquisition of imprinting. BMC Evol Biol 11:244.

Suzuki S, Renfree MB, Pask AJ, Shaw G, Kobayashi S, Kohda T, Kaneko-Ishino T, Ishino F (2005) Genomic imprinting of *IGF2*, *p57^{KIP2}* and *PEG1/MEST* in a marsupial, the tammar wallaby. Mechanisms of development 122:213-222.

Suzuki S, Fujisawa D, Hashimoto K, Asano T, Maimaiti M, Matsuo K, Tanahashi Y, Mukai T, Fujieda K (2010) Partial paternal uniparental disomy of chromosome 6 in monozygotic twins with transient neonatal diabetes mellitus and macroglossia. Clin Genet 78:580-584.

Swaney WT, Curley JP, Champagne FA, Keverne EB (2007) Genomic imprinting mediates sexual experience-dependent olfactory learning in male mice. Proc Natl Acad Sci U S A 104:6084-6089.

Swaney WT, Curley JP, Champagne FA, Keverne EB (2008) The paternally expressed gene Peg3 regulates sexual experience-dependent preferences for estrous odors. Behav Neurosci 122:963-973.

Swerdlow NR, Braff DL, Taaid N, Geyer MA (1994) Assessing the validity of an animal model of deficient sensorimotor gating in schizophrenic patients. Archives of general psychiatry 51:139.

Swerdlow NR, Shoemaker JM, Platten A, Pitcher L, Goins J, Auerbach PP (2004) Heritable differences in the dopaminergic regulation of sensorimotor gating. Psychopharmacology (Berl) 174:441-451.

Swerdlow NR, Shoemaker JM, Bongiovanni MJ, Neary AC, Tochen LS, Saint Marie RL (2005) Reduced startle gating after D1 blockade: effects of concurrent D2 blockade. Pharmacology Biochemistry and Behavior 82:293-299.

Szabó PE, Pfeifer GP (2012) H3K9me2 attracts PGC7 in the zygote to prevent Tet3-mediated oxidation of 5-methylcytosine. Journal of molecular cell biology 4:427-429.

Takahashi K, Nakayama K-i, Nakayama K (2000) Mice lacking a CDK inhibitor, p57Kip2, exhibit skeletal abnormalities and growth retardation. Journal of biochemistry 127:73-83.

Takikawa S, Wang X, Ray C, Vakulenko M, Bell FT, Li X (2013) Human and mouse ZFP57 proteins are functionally interchangeable in maintaining genomic imprinting at multiple imprinted regions in mouse ES cells. Epigenetics 8:1268.

Tateishi Y, Matsumoto A, Kanie T, Hara E, Nakayama K, Nakayama KI (2012) Development of mice without Cip/Kip CDK inhibitors. Biochemical and biophysical research communications 427:285-292.

Teegarden SL, Scott AN, Bale TL (2009) Early life exposure to a high fat diet promotes long-term changes in dietary preferences and central reward signaling. Neuroscience 162:924-932.

Terroni PL, Anthony FW, Hanson MA, Cagampang FRA (2005) Expression of agouti-related peptide, neuropeptide Y, pro-opiomelanocortin and the leptin receptor isoforms in fetal mouse brain from pregnant dams on a protein-restricted diet. Molecular Brain Research 140:111-115.

Thakur N, Tiwari VK, Thomassin H, Pandey RR, Kanduri M, Göndör A, Grange T, Ohlsson R, Kanduri C (2004) An antisense RNA regulates the

bidirectional silencing property of the Kcnq1 imprinting control region. Mol Cell Biol 24:7855-7862.
Thompson D, Martini L, Whistler JL (2010) Altered ratio of D1 and D2 dopamine receptors in mouse striatum is associated with behavioral sensitization to cocaine. PLoS One 5:e11038.
Thomsen M, Hall FS, Uhl GR, Caine SB (2009) Dramatically decreased cocaine self-administration in dopamine but not serotonin transporter knock-out mice. J Neurosci 29:1087-1092.
Thomson JA, Solter D (1988) The developmental fate of androgenetic, parthenogenetic, and gynogenetic cells in chimeric gastrulating mouse embryos. Genes Dev 2:1344-1351.
Thorre K, Sarre S, Smolders I, Ebinger G, Michotte Y (1998) Dopaminergic regulation of serotonin release in the substantia nigra of the freely moving rat using microdialysis. Brain research 796:107-116.
Thorvaldsen JL, Duran KL, Bartolomei MS (1998) Deletion of the H19 differentially methylated domain results in loss of imprinted expression of H19 and Igf2. Genes Dev 12:3693-3702.
Tidey JW, Miczek KA (1996) Social defeat stress selectively alters mesocorticolimbic dopamine release: an in vivo microdialysis study. Brain research 721:140-149.
Tomizawa S-i, Kobayashi H, Watanabe T, Andrews S, Hata K, Kelsey G, Sasaki H (2011) Dynamic stage-specific changes in imprinted differentially methylated regions during early mammalian development and prevalence of non-CpG methylation in oocytes. Development 138:811-820.
Tonkiss J, Shukitt-Hale B, Formica RN, Rocco FJ, Galler JR (1990) Prenatal protein malnutrition alters response to reward in adult rats. Physiol Behav 48:675-680.
Tournier BB, Ginovart N (2014) Repeated but not acute treatment with (9)-tetrahydrocannabinol disrupts prepulse inhibition of the acoustic startle: Reversal by the dopamine D2/3 receptor antagonist haloperidol. Eur Neuropsychopharmacol 24:1415-1423.
Tran DA, Bai AY, Singh P, Wu X, Szabó PE (2013) Characterization of the imprinting signature of mouse embryo fibroblasts by RNA deep sequencing. Nucleic acids research:gkt1042.
Trujillo ML, Spuch C, Carro E, Senaris R (2011) Hyperphagia and central mechanisms for leptin resistance during pregnancy. Endocrinology 152:1355-1365.
Tunster SJ, Tycko B, John RM (2010) The imprinted Phlda2 gene regulates extraembryonic energy stores. Mol Cell Biol 30:295-306.
Tunster SJ, Van de Pette M, John RM (2011) Fetal overgrowth in the Cdkn1c mouse model of Beckwith-Wiedemann syndrome. Dis Model Mech 4:814-821.
Tunster SJ, Jensen AB, John RM (2013) Imprinted genes in mouse placental development and the regulation of fetal energy stores. Reproduction 145:R117-R137.
Tunster SJ, Van De Pette M, John RM (2014) Isolating the role of elevated Phlda2 in asymmetric late fetal growth restriction. Dis Model Mech:dmm. 017079.

Turner CLS, Mackay DM, Callaway JLA, Docherty LE, Poole RL, Bullman H, Lever M, Castle BM, Kivuva EC, Turnpenny PD (2010) Methylation analysis of 79 patients with growth restriction reveals novel patterns of methylation change at imprinted loci. European Journal of Human Genetics 18:648-655.

Tury A, Mairet-Coello G, DiCicco-Bloom E (2011) The cyclin-dependent kinase inhibitor p57Kip2 regulates cell cycle exit, differentiation, and migration of embryonic cerebral cortical precursors. Cereb Cortex 21:1840-1856.

Úbeda F, Gardner A (2010) A model for genomic imprinting in the social brain: juveniles. Evolution 64:2587-2600.

Úbeda F, Gardner A (2011) A model for genomic imprinting in the social brain: Adults. Evolution 65:462-475.

Umlauf D, Goto Y, Cao R, Cerqueira F, Wagschal A, Zhang Y, Feil R (2004) Imprinting along the Kcnq1 domain on mouse chromosome 7 involves repressive histone methylation and recruitment of Polycomb group complexes. Nat Genet 36:1296-1300.

Usiello A, Baik JH, Rouge-Pont F, Picetti R, Dierich A, LeMeur M, Piazza PV, Borrelli E (2000) Distinct functions of the two isoforms of dopamine D2 receptors. Nature 408:199-203.

Van den Hove DL, Steinbusch HW, Scheepens A, Van de Berg WD, Kooiman LA, Boosten BJ, Prickaerts J, Blanco CE (2006) Prenatal stress and neonatal rat brain development. Neuroscience 137:145-155.

van den Hove DL, Jakob SB, Schraut KG, Kenis G, Schmitt AG, Kneitz S, Scholz CJ, Wiescholleck V, Ortega G, Prickaerts J, Steinbusch H, Lesch KP (2011) Differential effects of prenatal stress in 5-Htt deficient mice: towards molecular mechanisms of gene x environment interactions. PLoS One 6:e22715.

van der Heijden GW, Ramos L, Baart EB, van den Berg IM, Derijck AAHA, van der Vlag J, Martini E, de Boer P (2008) Sperm-derived histones contribute to zygotic chromatin in humans. BMC Dev Biol 8:34.

van der Plasse G, Schrama R, van Seters SP, Vanderschuren LJMJ, Westenberg HGM (2012) Deep brain stimulation reveals a dissociation of consummatory and motivated behaviour in the medial and lateral nucleus accumbens shell of the rat. PLoS One 7:e33455.

Van Loo PLP, Kruitwagen C, Van Zutphen LFM, Koolhaas JM, Baumans V (2000) Modulation of aggression in male mice: influence of cage cleaning regime and scent marks. Animal Welfare 9:281-295.

van Os J, Selten JP (1998) Prenatal exposure to maternal stress and subsequent schizophrenia. The May 1940 invasion of The Netherlands. Br J Psychiatry 172:324-326.

Van Tol HH, Bunzow JR, Guan HC, Sunahara RK, Seeman P, Niznik HB, Civelli O (1991) Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. Nature 350:610-614.

Vargas-Perez H, Sellings L, Grieder T, Diaz JL (2009) Social dominance rank influences wheel running behavior in mice. Neurosci Lett 457:137-140.

Varmuza S, Mann M (1994) Genomic imprinting—defusing the ovarian time bomb. Trends in Genetics 10:118-123.

Vazin T, Becker KG, Chen J, Spivak CE, Lupica CR, Zhang Y, Worden L, Freed WJ (2009) A novel combination of factors, termed SPIE, which promotes

dopaminergic neuron differentiation from human embryonic stem cells. PLoS One 4:e6606.
Verona RI, Mann MRW, Bartolomei MS (2003) Genomic imprinting: intricacies of epigenetic regulation in clusters. Annual review of cell and developmental biology 19:237-259.
Vollenweider FX, Csomor PA, Knappe B, Geyer MA, Quednow BB (2007) The effects of the preferential 5-HT2A agonist psilocybin on prepulse inhibition of startle in healthy human volunteers depend on interstimulus interval. Neuropsychopharmacology 32:1876-1887.
Vrang N, Meyre D, Froguel P, Jelsing J, Tang-Christensen M, Vatin V, Mikkelsen JD, Thirstrup K, Larsen LK, Cullberg KB, Fahrenkrug J, Jacobson P, Sjostrom L, Carlsson LM, Liu Y, Liu X, Deng HW, Larsen PJ (2010) The imprinted gene neuronatin is regulated by metabolic status and associated with obesity. Obesity (Silver Spring) 18:1289-1296.
Vucetic Z, Reyes TM (2010) Central dopaminergic circuitry controlling food intake and reward: implications for the regulation of obesity. Wiley Interdiscip Rev Syst Biol Med 2:577-593.
Vucetic Z, Kimmel J, Reyes TM (2011) Chronic high-fat diet drives postnatal epigenetic regulation of mu-opioid receptor in the brain. Neuropsychopharmacology 36:1199-1206.
Vucetic Z, Carlin JL, Totoki K, Reyes TM (2012) Epigenetic dysregulation of the dopamine system in diet-induced obesity. J Neurochem 120:891-898.
Vucetic Z, Kimmel J, Totoki K, Hollenbeck E, Reyes TM (2010a) Maternal high-fat diet alters methylation and gene expression of dopamine and opioid-related genes. Endocrinology 151:4756-4764.
Vucetic Z, Totoki K, Schoch H, Whitaker KW, Hill-Smith T, Lucki I, Reyes TM (2010b) Early life protein restriction alters dopamine circuitry. Neuroscience 168:359-370.
Wadhwa PD, Sandman CA, Porto M, Dunkel-Schetter C, Garite TJ (1993) The association between prenatal stress and infant birth weight and gestational age at birth: a prospective investigation. American journal of obstetrics and gynecology 169:858-865.
Wainwright P, Stefanescu R (1983) Prenatal protein deprivation increases defects of the corpus callosum in BALBc laboratory mice. Exp Neurol 81:694-702.
Walker QD, Cabassa J, Kaplan KA, Li S-T, Haroon J, Spohr HA, Kuhn CM (2001) Sex differences in cocaine-stimulated motor behavior: disparate effects of gonadectomy. Neuropsychopharmacology 25:118-130.
Wang F, Zhu J, Zhu H, Zhang Q, Lin Z, Hu H (2011a) Bidirectional control of social hierarchy by synaptic efficacy in medial prefrontal cortex. Science 334:693-697.
Wang J, Mager J, Schnedier E, Magnuson T (2002) The mouse PcG gene eed is required for Hox gene repression and extraembryonic development. Mamm Genome 13:493-503.
Wang W, Nitulescu I, Lewis JS, Lemos JC, Bamford IJ, Posielski NM, Storey GP, Phillips PEM, Bamford NS (2013) Overinhibition of corticostriatal activity following prenatal cocaine exposure. Annals of neurology 73:355-369.

Wang X, Soloway PD, Clark AG (2011b) A survey for novel imprinted genes in the mouse placenta by mRNA-seq. Genetics 189:109-122.
Ward HE, Johnson EA, Salm AK, Birkle DL (2000) Effects of prenatal stress on defensive withdrawal behavior and corticotropin releasing factor systems in rat brain. Physiol Behav 70:359-366.
Ward IL, Stehm KE (1991) Prenatal stress feminizes juvenile play patterns in male rats. Physiol Behav 50:601-605.
Wassum KM, Ostlund SB, Balleine BW, Maidment NT (2011) Differential dependence of Pavlovian incentive motivation and instrumental incentive learning processes on dopamine signaling. Learning & Memory 18:475-483.
Watabe-Uchida M, Zhu L, Ogawa SK, Vamanrao A, Uchida N (2012) Whole-brain mapping of direct inputs to midbrain dopamine neurons. Neuron 74:858-873.
Weinstock M (2008) The long-term behavioural consequences of prenatal stress. Neurosci Biobehav Rev 32:1073-1086.
Weksberg R, Shuman C, Smith AC (2005) Beckwith–Wiedemann syndrome. In: American Journal of Medical Genetics Part C: Seminars in Medical Genetics, pp 12-23: Wiley Online Library.
Weksberg R, Nishikawa J, Caluseriu O, Fei Y-L, Shuman C, Wei C, Steele L, Cameron J, Smith A, Ambus I (2001) Tumor development in the Beckwith–Wiedemann syndrome is associated with a variety of constitutional molecular 11p15 alterations including imprinting defects of KCNQ1OT1. Hum Mol Genet 10:2989-3000.
Wersinger SR, Rissman EF (2000) Dopamine activates masculine sexual behavior independent of the estrogen receptor alpha. J Neurosci 20:4248-4254.
Westbury J, Watkins M, Ferguson-Smith AC, Smith J (2001) Dynamic temporal and spatial regulation of the cdk inhibitor p57< sup> kip2</sup> during embryo morphogenesis. Mechanisms of development 109:83-89.
Wilkins JF (2014) Costs and consequences of the conflict over infant sleep. Evolution, medicine, and public health 2014:63-64.
Wilkins JF, Haig D (2003) Inbreeding, maternal care and genomic imprinting. Journal of Theoretical Biology 221:559-564.
Wilkinson LS, Davies W, Isles AR (2007) Genomic imprinting effects on brain development and function. Nat Rev Neurosci 8:832-843.
Williamson CM, Turner MD, Ball ST, Nottingham WT, Glenister P, Fray M, Tymowska-Lalanne Z, Plagge A, Powles-Glover N, Kelsey G (2006) Identification of an imprinting control region affecting the expression of all transcripts in the Gnas cluster. Nat Genet 38:350-355.
Wise RA (1998) Drug-activation of brain reward pathways. Drug and alcohol dependence 51:13-22.
Wise RA (2009) Roles for nigrostriatal—not just mesocorticolimbic—dopamine in reward and addiction. Trends in neurosciences 32:517-524.
Wolf JB (2013) Evolution of genomic imprinting as a coordinator of coadapted gene expression. Proceedings of the National Academy of Sciences 110:5085-5090.
Wolf JB, Hager R (2006) A maternal–offspring coadaptation theory for the evolution of genomic imprinting. PLoS biology 4:e380.

Wood AJ, Oakey RJ (2006) Genomic imprinting in mammals: emerging themes and established theories. PLoS Genet 2:e147.
Wood MD, Hiura H, Tunster SJ, Arima T, Shin J-Y, Higgins MJ, John RM (2010) Autonomous silencing of the imprinted Cdkn1c gene in stem cells. Epigenetics 5:214-221.
Woodfine K, Huddleston JE, Murrell A (2011) Quantitative analysis of DNA methylation at all human imprinted regions reveals preservation of epigenetic stability in adult somatic tissue. Epigenetics Chromatin 4:1.
Wozniak DF, Cicero TJ, Kettinger L, 3rd, Meyer ER (1991) Paternal alcohol consumption in the rat impairs spatial learning performance in male offspring. Psychopharmacology (Berl) 105:289-302.
Wright RL, Gilmour G, Dwyer DM (2013) Microstructural analysis of negative anticipatory contrast: A reconsideration of the devaluation account. Learn Behav 41:353-359.
Wutz A, Smrzka OW, Schweifer N, Schellander K, Wagner EF, Barlow DP (1997) Imprinted expression of the Igf2r gene depends on an intronic CpG island. Nature 389:745-749.
Wykes SM, Krawetz SA (2003) The structural organization of sperm chromatin. Journal of Biological Chemistry 278:29471-29477.
Wyvell CL, Berridge KC (2000) Intra-accumbens amphetamine increases the conditioned incentive salience of sucrose reward: enhancement of reward "wanting" without enhanced "liking" or response reinforcement. The Journal of neuroscience 20:8122-8130.
Wyvell CL, Berridge KC (2001) Incentive sensitization by previous amphetamine exposure: increased cue-triggered "wanting" for sucrose reward. The Journal of neuroscience 21:7831-7840.
Yan Y, Frisen J, Lee MH, Massague J, Barbacid M (1997) Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. Genes Dev 11:973-983.
Ye W, Mairet-Coello G, Pasoreck E, Dicicco-Bloom E (2009) Patterns of p57Kip2 expression in embryonic rat brain suggest roles in progenitor cell cycle exit and neuronal differentiation. Dev Neurobiol 69:1-21.
Yoneda T, Saitou K, Asano H, Mizushige T, Matsumura S, Eguchi A, Manabe Y, Tsuzuki S, Inoue K, Fushiki T (2009) Assessing palatability of long-chain fatty acids from the licking behavior of BALB/c mice. Physiol Behav 96:735-741.
Yoon B, Herman H, Hu B, Park YJ, Lindroth A, Bell A, West AG, Chang Y, Stablewski A, Piel JC (2005) Rasgrf1 imprinting is regulated by a CTCF-dependent methylation-sensitive enhancer blocker. Mol Cell Biol 25:11184-11190.
Yoon BJ, Herman H, Sikora A, Smith LT, Plass C, Soloway PD (2001) Regulation of DNA methylation of Rasgrf1. Nat Genet 30:92-96.
Yu Q, Teixeira CM, Mahadevia D, Huang Y, Balsam D, Mann JJ, Gingrich JA, Ansorge MS (2014) Dopamine and serotonin signaling during two sensitive developmental periods differentially impact adult aggressive and affective behaviors in mice. Mol Psychiatry.
Yu S, Yu D, Lee E, Eckhaus M, Lee R, Corria Z, Accili D, Westphal H, Weinstein LS (1998) Variable and tissue-specific hormone resistance in heterotrimeric Gs protein alpha-subunit (Gsalpha) knockout mice is due to tissue-

specific imprinting of the gsalpha gene. Proc Natl Acad Sci U S A 95:8715-8720.
Yu S, Gavrilova O, Chen H, Lee R, Liu J, Pacak K, Parlow AF, Quon MJ, Reitman ML, Weinstein LS (2000) Paternal versus maternal transmission of a stimulatory G-protein α subunit knockout produces opposite effects on energy metabolism. The Journal of clinical investigation 105:615-623.
Zagron G, Weinstock M (2006) Maternal adrenal hormone secretion mediates behavioural alterations induced by prenatal stress in male and female rats. Behav Brain Res 175:323-328.
Zetterström RH, Williams R, Perlmann T, Olson L (1996) Cellular expression of the immediate early transcription factors Nurr1 and NGFI-B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. Molecular Brain Research 41:111-120.
Zetterström RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T (1997) Dopamine neuron agenesis in Nurr1-deficient mice. Science 276:248-250.
Zhang H, Zeitz MJ, Wang H, Niu B, Ge S, Li W, Cui J, Wang G, Qian G, Higgins MJ (2014) Long noncoding RNA-mediated intrachromosomal interactions promote imprinting at the Kcnq1 locus. The Journal of cell biology 204:61-75.
Zhang LI, Poo M-m (2001) Electrical activity and development of neural circuits. Nature Neuroscience 4:1207-1214.
Zhang M, Balmadrid C, Kelley AE (2003) Nucleus accumbens opioid, GABaergic, and dopaminergic modulation of palatable food motivation: contrasting effects revealed by a progressive ratio study in the rat. Behav Neurosci 117:202-211.
Zhang P, Liegeois NJ, Wong C, Finegold M, Hou H, Thompson JC, Silverman A, Harper JW, DePinho RA, Elledge SJ (1997) Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. Nature 387:151-158.
Zhao R, Yang HY, Shin J, Phan L, Fang L, Che TF, Su CH, Yeung SC, Lee MH (2013) CDK inhibitor p57 (Kip2) is downregulated by Akt during HER2-mediated tumorigenicity. Cell Cycle 12:935-943.
Zhou QY, Grandy DK, Thambi L, Kushner JA, Van Tol HH, Cone R, Pribnow D, Salon J, Bunzow JR, Civelli O (1990) Cloning and expression of human and rat D1 dopamine receptors. Nature 347:76-80.
Zhuang X, Oosting RS, Jones SR, Gainetdinov RR, Miller GW, Caron MG, Hen R (2001) Hyperactivity and impaired response habituation in hyperdopaminergic mice. Proceedings of the National Academy of Sciences 98:1982-1987.
Zuo X, Sheng J, Lau H-T, McDonald CM, Andrade M, Cullen DE, Bell FT, Iacovino M, Kyba M, Xu G (2012) Zinc finger protein ZFP57 requires its co-factor to recruit DNA methyltransferases and maintains DNA methylation imprint in embryonic stem cells via its transcriptional repression domain. Journal of Biological Chemistry 287:2107-2118.

Appendix A

Table A.1: qPCR results for *Cdkn1c*^{BACx1} line. Where variables passed Shapiro-Wilk test for normality, t statistic is presented, where variable failed test for normality Mann-Whitney Z scores are presented. Tests where there was a significant effect of genotype are indicated in bold.

Region	Gene	n=(wt, tg)	Statistics
Frontal cortex	***Drd1***	**n=(8,5)**	**Z=-2.049, p=0.04**
	Drd2	**n=(8,5)**	**t(11)=-2.423, p=0.034**
	Th	n=(8,5)	t(11)=-0.685, p=0.507
	Dat	n=(4,4)	t(11)=-1.075, p=0.308
Dorsal striatum	*Drd1*	n=(4,4)	t(6)=0.54, p=0.609
	Drd2	n=(4,4)	t(6)=0.04, p=0.970
	Th	n=(4,4)	Z=-0.866, p=0.486
	Dat	**n=(4,3)**	**t(5)=4.504, p=0.006**
Ventral striatum	*Drd1*	n=(7,4)	t(9)=1.097, p=0.301
	Drd2	n=(7,4)	t(9)=0.615, p=0.554
	Th	n=(7,4)	t(9)=-1.075, p=0.075
Hypothalamus	*Drd1*	n=(4,3)	t(5)=1.102, p=0.321
	Drd2	n=(4,3)	t(5)=1.695, p=0.151
	Th	n=(5,3)	t(6)=-0.426, p=0.685

Table A.2: qPCR results for *Cdkn1c*^{BACLacZ} line. Where variables passed Shapiro-Wilk test for normality, t statistic is presented, where variable failed test for normality Mann-Whitney Z scores are presented.

Region	Gene	n=(wt, tg)	Statistics
Frontal cortex	*Drd1*	n=(7,5)	Z=-0.406, p=0.685
	Drd2	n=(7,5)	Z=-0.568, p=0.57
	Th	n=(7,5)	t(10)=-0.329, p=749
	Dat	n=(5,3)	Z=-0.745, p=0.571
Dorsal striatum	*Drd1*	n=(4,5)	t(7)=-0.916, p=0.39
	Drd2	n=(4,5)	t(7)=-0.915, p=0.391
	Th	n=(3,5)	t(6)=-2.247, p=0.066
	Dat	n=(4,6)	t(8)=-0.1, p=0.923
Ventral striatum	*Drd1*	n=(3,4)	t(5)=-0.92, p=0.4
	Drd2	n=(3,4)	t(5)=-0.22, p=0.834
	Th	n=(3,4)	t(5)=0.088, p=0.933
Hypothalamus	*Drd1*	n=(4,3)	t(5)=1.959, p=0.107
	Drd2	n=(4,3)	t(5)=-0.374, p=0.724
	Th	n=(5,3)	t(6)=-0.447, p=0.671

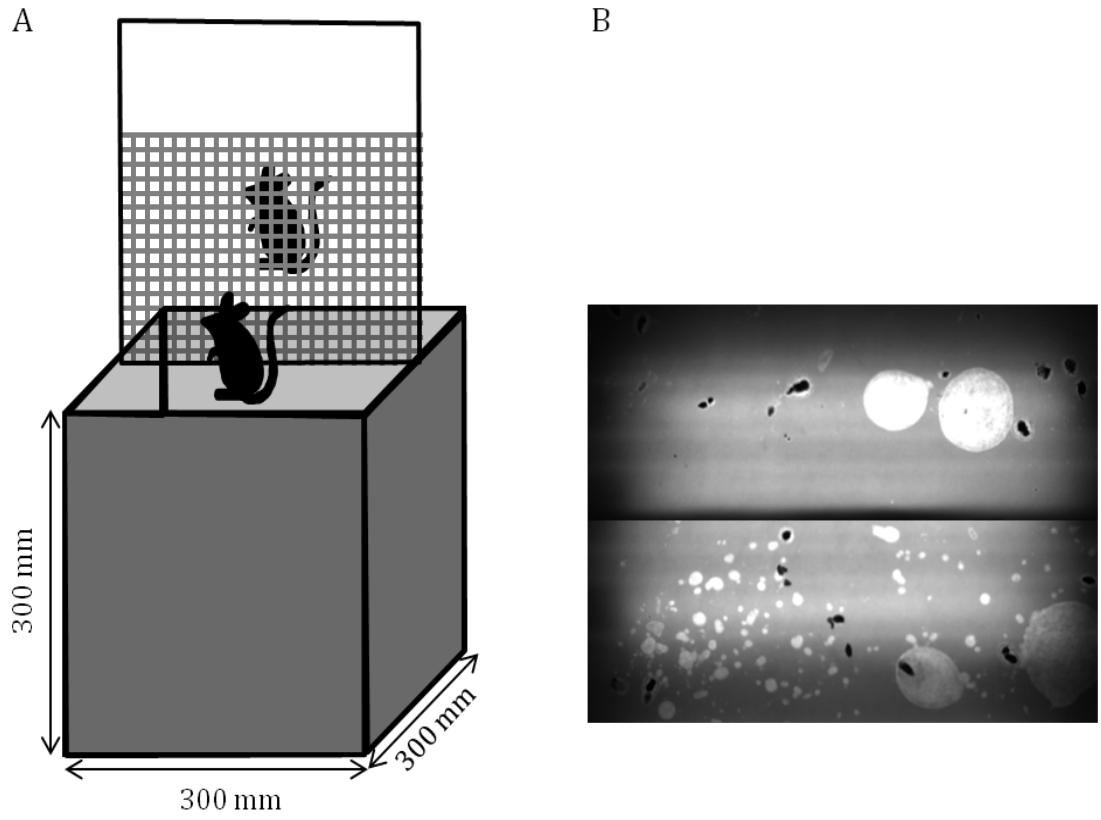
Table A.3: HPLC result for *Cdkn1c*^{BACx1} line. F values shown are adjusted for multiple comparisons with a region. All variables passed Shapiro-Wilk test for normality. n *Cdkn1c*^{BACx1}= 9, n wt littermates=7. Tests where there was a significant effect of genotype are indicated in bold.

Region	Metabolite	$F_{1,14}$=	p=
Frontal cortex	Noreadrenaline	0.308	0.588
	Dopamine	1.079	0.317
	Serotonin	3.696	0.075
	DOPAC	0.618	0.445
	5HIAA	2.919	0.11
Dorsal striatum	Noreadrenaline	0.002	0.969
	Dopamine	**5.279**	**0.038**
	Serotonin	**5.102**	**0.04**
	DOPAC	0.104	0.751
	5HIAA	1.039	0.325
Ventral striatum	Noreadrenaline	0.535	0.477
	Dopamine	0.007	0.934
	Serotonin	6.686	**0.022**
	DOPAC	0.395	0.54
	5HIAA	0.006	0.939
Hypothalamus	Noreadrenaline	0.551	0.47
	Dopamine	2.222	0.158
	Serotonin	0.725	0.409
	DOPAC	**4.99**	**0.042**
	5HIAA	**6.948**	**0.02**

Table A.4: HPLC result for *Cdkn1c*^{BACLacZ} line. F values shown are adjusted for multiple comparisons with a region. All variables passed Shapiro-Wilk test for normality. n *Cdkn1c*^{BACLacZ}= 12, n wt littermates=4			
Region	Metabolite	$F_{1,14}$=	p=
Frontal cortex	Noreadrenaline	0.62	0.443
	Dopamine	0.853	0.371
	Serotonin	0.17	0.686
	DOPAC	0.687	0.421
	5HIAA	0.067	0.799
Dorsal striatum	Noreadrenaline	0.591	0.455
	Dopamine	0.481	0.499
	Serotonin	0.178	0.679
	DOPAC	1.428	0.252
	5HIAA	0.556	0.468
Ventral striatum	Noreadrenaline	0.237	0.634
	Dopamine	0.349	0.564
	Serotonin	1.471	0.245
	DOPAC	0.554	0.469
	5HIAA	1.316	0.271
Hypothalamus	Noreadrenaline	0.084	0.777
	Dopamine	0.05	0.827
	Serotonin	0.075	0.788
	DOPAC	0.23	0.639
	5HIAA	0.566	0.464

Appendix B

Figure B.1: Schematic of urine marking apparatus (A) and typical subordinate (top) and dominant (bottom) scent marking pattern, visualised under UV light (B).



Appendix C: Details of diet for Chapter 7