The effect of Interleukin-6 and maternal high fat diet on developmental gene expression in the embryonic mouse brain

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Abstract

Perturbations in the maternal environment during gestation can have serious health implications for the resulting child later in life. Maternal inflammation is one disruption to the maternal environment that is linked to several disease states in adult life. For example, in mice it has been shown that activation of the immune system and increased levels of the cytokines during gestation affects the development of brain areas involved in social interaction and memory, and results in a schizophrenia-like phenotype (Baharnoori et al., 2009; Hsiao and Patterson, 2011). In humans this finding is supported by epidemiological studies that demonstrate an association between maternal infection and the development of schizophrenia (Brown et al., 2004b).

Obesity is another disease state that may be caused by maternal inflammation. A correlation has been clearly shown in humans and rodent models between maternal obesity during pregnancy, and obesity in the offspring (Chen et al., 2008; Howie et al., 2009; Whitaker, 2004; Wu and Suzuki, 2006) but the mechanism behind this is unknown. It has recently emerged that obesity is accompanied by a chronic state of low grade inflammation; obese individuals display significantly elevated levels of cytokines (Calabro et al., 2009). Maternal inflammation could therefore mediate the predisposition of offspring to obesity later in life by affecting the development of brain areas that regulate body weight, just as perturbations may predispose children to schizophrenia.

The hippocampus is one important brain region involved in social interaction and memory, and has been implicated in the development of schizophrenia (White et al., 2008). The arcuate nucleus is a different region that regulates bodyweight and it thus implicated in the development of obesity (Arora and Anubhuti, 2006). We therefore hypothesised that elevated levels of maternal cytokines disrupt correct embryonic development of the hippocampus and arcuate nucleus by affecting the expression of key developmental genes, and that this disruption programs the increased risk of offspring for neurological disease later in life. The cytokine Interleukin-6 (IL-6) seems to be a major player in the prenatal programming of both these disorders (Dahlgren et al., 2006; Smith et al., 2007) so we initially used an in vitro model in
which embryonic day 17.5 mouse brain was exposed to IL-6 at 100 ng/ml for 1 hour. The expression of genes encoding several developmentally important signalling molecules and their receptors were examined in the hippocampus and arcuate nucleus using quantitative PCR. These results were then replicated in vivo in the arcuate nucleus by creating a mouse model of maternal obesity.

We found that in vitro IL-6 treatment altered the expression of the key neurodevelopmental genes Netrin1, Dcc, Sonic hedgehog and Gli1 in the embryonic hippocampus and/or arcuate nucleus. This implicates a possible mechanism by which prenatal IL-6 exposure disrupts the expression of important neurodevelopmental genes during embryogenesis, resulting in abnormal brain development and leading to an increased risk of neurological disease later in life. In addition to this we created a mouse model of maternal obesity. We then used this model to show that the same gene expression changes that occurred in the arcuate nucleus in response to in vitro IL-6 treatment also happen in vivo in response to maternal obesity. These data are key in establishing a mechanism to explain how increased IL-6 levels in maternal obesity are able to programme offspring to obesity.

This research provides good evidence that fetal exposure to IL-6 during pregnancy changes the expression of important neurodevelopmental genes in the embryonic hippocampus and arcuate nucleus during prenatal development. Future research in this area has the potential to improve human health by creating a research-informed basis for human pregnancy health guidelines that will reduce the risk of these neurological disorders.
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Abbreviations

ActB    Beta Actin
AGRP    Agouti-related protein
bp      Base pairs
B2m     Beta-2 microglobulin
CART    Cocaine and amphetamine regulated transcript
cDNA    Complementary DNA
CpG     Cytosine-guanine dinucleotide
Dcc     Deleted in colorectal cancer
dNTP    Deoxyribose nucleotide
ERK     Extracellular signal-regulated kinase
Gli1    Glioma associated oncogene
HF      High fat
IL-6    Interleukin-6
IL-6R   Interleukin-6 receptor
JAK     Janus kinase
kb      Kilobases
LPS     Lipopolysaccharide
MIA     Maternal immune activation
MHF     Maternal high fat
NF-κB   Nuclear factor kappa-B
NPY     Neuropeptide Y
Ntn1    Netrin1
PCR     Polymerase chain reaction
Pgk1    Phosphoglycerate kinase 1
Poly IC  Polyinosinic:polycytidylic acid
POMC    Pro-opimelanocortin
qPCR    Quantitative PCR
Shh     Sonic hedgehog
SNP     Single nucleotide polymorphism
STAT    Signal transducers and activators of transcription
TNF-α   Tumour necrosis factor alpha
Chapter 1: Introduction
1.1 Fetal origins of adult health and disease

Throughout an individual’s life there are many times in which the environment’s interaction with their genome plays a role in their health and development. Perhaps the most important instance is at the very beginning of life. The interactions between the fetus and the maternal environment it is exposed to in utero coincide with a critical period of growth and development, so it has long been acknowledged that adverse life events experienced by a pregnant woman can have major effects on the fetus and their health in later life. The classic example focuses on pregnancies during the Dutch famine of 1944. At middle age, individuals who had been exposed to the famine in utero had a higher incidence of cardiovascular disease, obesity and glucose intolerance than individuals born the year before or after the famine (Painter et al., 2005; Roseboom et al., 2006). These observations in humans have been replicated in rodent studies showing that maternal under nutrition leads to offspring that in adult life develop hyperphagia, insulin resistance, hypertension and obesity (Krechowec et al., 2006; Vickers et al., 2000). Interestingly, a similar phenotype is seen in offspring exposed to an over-nourishing maternal environment (Howie et al., 2009).

However, it is not just what a mother is eating during pregnancy that is important. More subtle perturbations to maternal health have also been shown to greatly effect offspring health. Work in rodents has shown that maternal stress and anxiety can have a great effect on the brain development and behavioural phenotype of offspring (Sarkar et al., 2008). Human studies back this up: offspring of mothers exposed to stress are more at risk of a number of neurological problems, including depression, hyperactivity, attention deficits and drug abuse (Sarkar et al., 2008; Weinstock, 2005). Additionally, mounting evidence from both human and animal studies indicates that the developmental origins of polycystic ovarian syndrome, a disorder that manifests in women during adolescence, lie in prenatal exposure of the fetus to increased maternal androgens (Abbott et al., 2002; Xita and Tsatsoulis, 2006). Finally, inflammation during pregnancy has long been associated with increased risk to the offspring of mental illness.
1.2 Maternal inflammation and the fetal brain
The most well appreciated cause of inflammation during pregnancy is due to infection by a pathogen. However, it can also be present in the form of low grade chronic inflammation, such as is seen with certain inflammatory diseases and during obesity (Shoelson et al., 2007). During inflammation there is an increase in the maternal circulation of inflammatory cytokines to which the fetus is then at risk of being exposed. Some cytokines, such as interleukin-6 (IL-6), have been shown in the rodent model to cross both the placenta and blood-brain barrier (Banks et al., 1994; Dahlgren et al., 2006). Additionally mouse models of maternal immune activation have shown that increased levels of maternal IL-6 lead to increased placental production of IL-6 (Hsiao and Patterson, 2011). Maternal infection and inflammation have been linked to autism and the development of schizophrenia in adult life, and more recently an increased risk of childhood obesity (Challier et al., 2008; Patterson, 2009).

1.3: Schizophrenia and its prenatal origins
Schizophrenia is a severe psychiatric illness characterised by positive symptoms such as delusions and hallucinations, negative symptoms such as social withdrawal, apathy and depression, and a long duration (van Os and Kapur, 2009; White et al., 2008). Despite being formally defined over 100 years ago, very little is known about the aetiology of the disease (Tandon et al., 2008). The most concrete risk factor currently known in developing schizophrenia is a genetic predisposition. While genome wide association studies have been helpful in identifying rare, highly penetrant causative genetic variants, it has become increasingly clear that in the vast majority of cases, the aetiology of schizophrenia is a complex mixture of gene-environment interactions (Kang et al., 2011; van Haren et al., 2008).

Since the late 1980’s it has been acknowledged that perturbations to the prenatal environment, in particular maternal infection, play a major role in the development of this disorder. Many studies have reported an association between influenza infection during pregnancy and an increased risk to schizophrenia in offspring (Brown et al., 2004a; Izumoto et al., 1999; McGrath et al., 1994; Mednick et al., 1988). This association has also been shown in a variety of other maternal illnesses, such as rubella, and respiratory tract infections (Brown, 2006; Brown et al., 2001). However
none of these epidemiological studies in humans are able to prove causation or suggest a mechanism of how this predisposition occurs.

Because very different pathogens seem to cause the same phenotype, and since it is unlikely they are able to directly infect the fetus (Irving et al., 2000; Shi et al., 2005; Williams and Mackenzie, 1977), the maternal immune response has been implicated. Maternal immune activated (MIA) rodent models have been particularly helpful to test *in vivo* effects of the maternal immune system on the developing fetal brain. These models predominantly administrate either bacterial lipopolysaccharide (LPS) or double stranded viral RNA (poly IC) to pregnant rats or mice. This induces an immune response, increasing circulating levels of cytokines without the confounding effects of the introduction of a viral or bacterial pathogen itself. Offspring of these MIA mice have been reported to show a ‘schizophrenic-like’ phenotype, including deficits in prepulse inhibition (PPI), latent inhibition (LI), anxiety, locomotion and social interaction (Meyer et al., 2008b; Smith et al., 2007).

Interestingly the inflammatory cytokine IL-6 seems to play a significant role during MIA. Smith et al. (2007) found that use of an IL-6 receptor knock out mouse, or co-administration of an IL-6 antibody, with poly IC is able to fully prevent the ability of MIA to induce a schizophrenic-like phenotype. The researchers additionally found that sole injection of IL-6 during pregnancy was enough to cause PPI deficits in offspring. This was not the case for other inflammatory cytokines such as IL-1α, tumour necrosis factor α (TNFα) and interferon γ (IFNγ). Therefore it seems likely that IL-6 is directly affecting fetal brain development in the MIA model, an intriguing possibility considering IL-6 has been documented to have many neurotrophic roles (Spooren et al., 2011).
1.4 Schizophrenia and the hippocampus

Many regions of the brain have been associated with the pathophysiology of schizophrenia, but a strong body of evidence implicates the hippocampus as a key area. Firstly, many features of schizophrenia are deficits in functions carried out by the limbic system, which includes the hippocampus. These include both positive symptoms such as inability to correctly process sensory stimuli (resulting in delusions and hallucinations), as well as negative symptoms such as depression and problems with social interaction (White et al., 2008). Additionally, there is a high incidence of schizophrenia in individuals suffering from temporal lobe epilepsy, a disorder which results in hippocampal damage (Gothelf et al., 2000). MRI studies have also demonstrated a decrease in hippocampal size in newly diagnosed schizophrenic patients, indicating this feature is associated with the disorder and not a result of antipsychotic medication (Velakoulis et al., 1999; Wood et al., 2001). Alterations in connections and interactions between the hippocampus and other brain regions, such as the prefrontal cortex, have also been described both in chronic and newly diagnosed patients (Benetti et al., 2009; Zhou et al., 2008).

In human schizophrenia cases, studies of hippocampal development and connections in patients is limited to brain scanning techniques. However several rodent models of schizophrenia have been used for further study in this field. For example, Sigurdsson et al. (2010) created a genetic mouse model of the known 22q11.2 microdeletion risk allele. In this model they found reduced synchrony between hippocampal and prefrontal neurons, implicating a deficit with connectivity on a single cellular level. Another study (Baharnoori et al., 2009) used the LPS-induced MIA schizophrenia model in the rat and specifically looked at dendritic morphology of neurons in the hippocampus and prefrontal cortex. They found that MIA affected the morphology and temporal development of pyramidal neurons in these regions in the early postnatal days and with some abnormalities persisting into adulthood.

Both human and rodent data suggest that the hippocampus, and its connections with other brain regions, is very important in the pathology of schizophrenia. However it remains unclear how exactly it is being disrupted in this disorder.
1.5 Development of the hippocampus

In order to understand the role of the hippocampus in schizophrenia we must first be aware of aspects of normal hippocampal development. During neural development neurons make connections both within and between brain regions. They do this by projecting out axons, which move along a fixed path, sometimes along a relatively long distance, and make contact with a specific target. The process of guiding developing axons is governed by ‘axon guidance factors’ (Chedotal and Richards, 2010). These are molecules that are able to attract or repulse the leading edge of the migrating axon in order to keep it on its correct path. If the expression of these axon guidance factors is disrupted, for example in the hippocampus, then this would in turn disrupt the correct wiring of this area, potentially resulting in a neurological disorder such as schizophrenia.

The mature hippocampus is a complex and dynamic structure within the brain and many guidance factors are involved in creating the correct pattern of neuronal wiring. One important axon guidance molecule in this region is Netrin1 (Ntn1). Ntn1 works to attract axons that express the receptor Dcc (see figure 1.1), both of which are expressed in the embryonic mouse hippocampus, particularly from embryonic day 14 (E14) to E18 (Barallobre et al., 2000; Shu et al., 2000). In 1996, Serifini et al. created a mouse line in which the Ntn1 gene was knocked out. Amongst the multitude of neurodevelopmental defects in this mouse, the authors described problems with the formation of the hippocampal commissure due to the absence of axons being guided into this area. Further investigation into the role of Ntn1 in the hippocampus has shown its importance in forming correct connections both within the hippocampus, and between the hippocampus and other neural regions (Barallobre et al., 2000; Muramatsu et al., 2010; Pascual et al., 2004).
Figure 1.1: The importance of Netrin1 and its receptor Dcc in the guidance of Netrin1 responsive axons. (a) The receptor Dcc is required to attract axons towards a Ntn1 gradient. (b) If the expression of Ntn1 or Dcc is disrupted then the developing axon will not be guided towards its target. *Diagram adapted from Arakawa (2004)*
Another molecule that may be important in hippocampal development is Sonic Hedgehog (Shh), a member of the hedgehog family of signalling molecules. It has a diverse set of roles within the developing nervous system ranging from neural patterning (Ruiz i Altaba et al., 2002), to cell fate (reviewed in Martí and Bovolenta (2002)) and more recently axon guidance (Bourikas et al., 2005). It also plays a role in the adult nervous system, particularly in hippocampal neurogenesis (Breunig et al., 2008; Lai et al., 2003). Shh signals through the receptor Patched (Ptc), ultimately translocating the transcription factor Gli1 to the cell nucleus to mediate gene expression (Ruiz i Altaba et al., 2002)(see figure 1.2). Although currently there is no direct evidence of prenatal Shh or Gli1 expression in the hippocampus, Shh is present in this region at birth (Favaro et al., 2009). Additionally, Shh has been shown at the midline to collaborate with Ntn1 in axon guidance (Charron et al., 2003).

Therefore Ntn1 and Shh and their associated signalling pathways are both good candidates for how hippocampal development might be going wrong in schizophrenia. Of course, both these molecules are expressed throughout the brain during embryonic development. Thus, if maternal inflammation is able to somehow disrupt their expression in the hippocampus, it could be doing so in multiple brain areas as well, leading to other neurological pathologies. One of these, although very different to schizophrenia, is childhood obesity.
**Figure 1.2: Shh signalling pathway.** Extracellular Shh activates Patched (Ptc), releasing its repression on Smoothened (Smo). This in turn activates the Gli1 transcription factor in the nucleus, which binds target genes and modulates their transcription. *Diagram adapted from Radtke and Raj (2003).*
1.6 Obesity and its prenatal origins

Obesity is one of the biggest health problems facing the Western world today (Armitage et al., 2008). As well as being debilitating in and of itself, it leads to many more serious conditions including type 2 diabetes, hypertension, stroke and cardiovascular disease (Kiess et al., 2001; Tao, 2005). Of greater concern is the rapid rise in childhood obesity seen within the last few decades, and the resulting increase in childhood type 2 diabetes (Howie et al., 2009). Being a Westernised country New Zealand has not escaped this epidemic, with 1 in 10 children aged 5 to 14 years old considered obese, and a further 20% overweight (Taylor, 2007). The picture is even poorer for Pacific Island communities where up to 45% of 2 to 5 year olds may be obese (Grant et al., 2004). Generally, childhood obesity occurs in a similar way to adult obesity with a higher rate of energy intake than energy expenditure (Deckelbaum and Williams, 2001). However, over the last couple of decades evidence has mounted that often the origins of childhood obesity may lie in early development. What we do not yet understand is how and why this might be occurring.

Epidemiological studies of obese mothers and their children highlight maternal obesity as a risk factor for obesity in offspring. For example, a retrospective cohort study conducted by Whitaker (2004) found that almost a quarter of children born to mothers who were obese during the early stages of pregnancy were obese themselves by age four, compared to nine percent from normal weight mothers. These observations were first made in humans, but since then a multitude of rodent studies have corroborated the findings and shown that changes occurring in utero seem to be very important in programming offspring obesity (Chang et al., 2008; Howie et al., 2009; Shankar et al., 2008).
1.7 Obesity as an inflammatory disease

Taken together, both animal and human findings show that the fetal risk of developing obesity is a U-shaped curve, with both maternal under- and over-nutrition conferring the most risk (Grattan, 2008) (See figure 1.3a). This same pattern is seen in terms the inflammatory response, as both maternal under- and over-nutrition increase certain inflammatory cytokine levels (Catalano et al., 2009; Clarke, 2010; Schmatz et al., 2010a; Shen et al., 2008) (see figure 1.3b). Just as in schizophrenia, maternal cytokines may be mediating predisposition of offspring to obesity later in life. Interestingly, there is some evidence from a MIA mouse model that metabolic problems associated with schizophrenia, such as obesity and type 2 diabetes, may also be caused by prenatal immune challenge (Pacheco-López et al., 2011).

Maternal obesity is associated with low-grade inflammation characterised by increases in the inflammatory cytokines IL-6, IL-1 and TNFα in both the maternal circulation and the placenta (Challier et al., 2008; Schmatz et al., 2010a). Of particular interest is the increase in IL-6, as circulating levels of IL-6 have been found to be 1.5 fold higher in pregnant woman who are obese, compared with their normal weight counterparts (Ramsay et al., 2002). Additionally, as previously mentioned (see section 1.2), IL-6 has the ability to cross the placenta to directly influence the fetal environment and stimulate placental IL-6 production (Dahlgren et al., 2006). Lastly, when IL-6 is injected into pregnant rats this results in offspring with increased adipose tissue and body weight (Dahlgren et al., 2006). However once again it seems unclear how IL-6, or maternal cytokines in general, might be predisposing offspring to obesity.
Figure 1.3a: Maternal body weight is associated with relative obesity risk in offspring. Maternal under and over nutrition confer the most risk of offspring to obesity, forming a U-shaped curve. *Diagram adapted from Grattan (2008)*

Figure 1.3b: Maternal body weight is associated with relative levels of inflammatory cytokines. Women under or over nourished during pregnancy have increased levels of certain inflammatory cytokines, forming a U-shaped curve.
1.8 Neuroendocrine control of body weight

In order to begin to answer how a child might be predisposed to obesity it is important to understand how body weight is normally regulated. The hypothalamus is a key area involved in the synthesis and response to neural, endocrine, and nutritional messages concerning body weight (Nobili et al., 2010). Of particular importance within the hypothalamus is an area called the arcuate nucleus. The arcuate nucleus is positioned so that peripheral signals travelling from the body can access it directly by bypassing the blood-brain barrier (Arora and Anubhuti, 2006). The neurons present in this area express one of two pairs of neuropeptides: POMC and CART that suppress appetite, or NPY and AGRP that stimulate appetite (Arora and Anubhuti, 2006). The POMC/CART and NPY/AGRP neurons present in the arcuate nucleus make connections with other important weight regulatory areas of the hypothalamus, including the paraventricular nucleus (PVN), the Dorsomedial hypothalamus and the lateral hypothalamic area (Arora and Anubhuti, 2006; Bouret et al., 2004). The relative levels of POMC/CART and NPY/AGRP received by neurons in these target nuclei determines if further signaling is required to begin or cease feeding (Grove et al., 2005) (see figure 1.4).
Figure 1.4: Regulation of body weight by hypothalamic neurons. Peripheral signals from all over the body either inhibit (red line) or activate (green arrow) the expression of POMC/CART or NPY/AGRP. The relative levels of these neuropeptides signal to second order neurons in the Paraventricular Nucleus (PVN), the Dorsomedial Hypothalamus (DMH) and the Lateral Hypothalamic Area (LHA) to adjust food intake.
1.9 Arcuate nucleus development and axon guidance

POMC/CART and NPY/AGRP neurons play such an integral role in controlling body weight it is easy to suppose that if the ways in which they make connections and communicate with other parts of the brain was disrupted this could lead to problems with weight regulation. How exactly the arcuate nucleus develops prenatally is only just starting to be understood. In primates, projections from arcuate neurons into the PVN are known to develop during the third trimester (Grove et al., 2005), so their development could well be perturbed by an over-nourishing maternal environment. The situation is less clear in rodent models. The majority of studies looking at arcuate development have focused on the early postnatal period, when most projections occur (Grove et al., 2005), but when their growth is no longer influenced by the maternal environment. However, arcuate projections have been detected at post-natal day one in rodents (Kagotani et al., 1989; Woodhams et al., 1985), indicating that the growth of the axons forming the projections occurs in utero. In addition, recent work within our lab has confirmed the embryonic development of arcuate-PVN projections from NPY neurons at embryonic day 15 in the mouse. Lastly, Padilla et al. (2010) have shown that POMC neurons are born about embryonic day 10.5, and NPY neurons about embryonic day 14.5, further supporting the hypothesis that their axonal projections are forming prior to birth. Although fewer in number, initial prenatal projections are important in normal neural development as they provide a scaffold over which projections that occur postnatally can develop (reviewed in Chedotal and Richards (2010). Therefore, if this early fetal circuitry was disrupted in the arcuate nucleus, these changes would be elaborated during post-natal development, thus affecting the weight regulatory mechanisms in the brain into adult life.

No one has yet established which guidance molecules are involved in guiding arcuate projections. However, Ntn1 is a good candidate as it is expressed along the ventral midline of the nervous system where it is heavily involved in patterning axonal projections (Killeen and Sybingco, 2008). Additionally, our lab has shown that the Ntn1 receptor Dcc is expressed in the arcuate nucleus at embryonic day 15.5 and 17.5 (unpublished). Therefore the correct expression levels and patterns of Ntn1 and Dcc are likely to be instrumental in the formation of fully functioning weight regulatory circuitry.
Shh is also a good candidate for playing a role in arcuate development. Investigations in our lab have shown that Shh protein borders on the arcuate nucleus at mid-late gestation (Chew-Ling Tan, unpublished data). Additionally, at E15 and E17 we have found expression of the Shh receptors Smo and Boc in the arcuate nucleus (Thomas Kim, unpublished data). Boc has been previously shown to play a role in Shh mediated axon guidance (Okada et al., 2006). Additionally, Yam et al. (2009) have shown that Smo is required for the induction of Src family kinase activity in response to Shh, a mechanism which is key in Shh mediated axon guidance.

1.10 Cytokine mediated changes in developmental gene expression

There is some evidence that the maternal immune system modulates expression of developmental genes within the fetal brain. For example, Meyer et al. (2008a) found fetal brain from MIA mice had altered expression in a number of genes involved in neuronal development, including Shh. As has already been discussed (see sections 1.3 and 1.7) the cytokine IL-6 is a good candidate as a major player in these changes, potentially leading to both schizophrenia and obesity. IL-6 binds the common signal transducer gp130, via its specific receptor IL-6Rα (Spooren et al., 2011). In this configuration it can influence gene expression in several ways (see figure 1.5). First via activation of the JAK/STAT pathway culminating in phosphorylation of the transcription factor STAT3 which translocates to the nucleus and modulates gene expression (Heinrich et al., 2003). This pathway is well known to be active in IL-6 signaling in the central nervous system (Spooren et al., 2011). Second, by activation of the transcription factor NF-κB, which then translocates to the nucleus and also affects expression of target genes (Ma et al., 2010; Wang et al., 2003). The other important IL-6 activated signaling cascade is the Erk1/2 pathway. This pathway also regulates transcription factor activity via phosphorylation, and so also has the ability to modulate expression of specific genes.
Figure 1.5: IL-6 signalling pathways. IL-6 binds IL-6R which activates gp130. This in turn activates three signalling pathways. (1) Phosphorylated SHP2 activates RAS which activates the ERK1/2 pathway. ERKs can phosphorylate certain transcription factors and therefore influence gene activity. (2) Activated gp130 releases inhibition of NF-κB by IκBα. NF-κB translocates to the nucleus and influences gene expression. (3) JAK1 phosphorylates Stat3 which forms homodimers and also translocates to the nucleus, binding target DNA and influencing gene expression. Diagram adapted from Spooren et al. (2011) and Ma et al. (2010).
1.11 Hypothesis and aims
We hypothesise that increased levels of maternal IL-6 affects the expression of key developmental genes in the embryonic arcuate nucleus and hippocampus. This then predisposes the offspring to disease in later life. We had several different aims to address in this hypothesis:

- **Aim 1**: To investigate whether treatment of mouse E17.5 hippocampus with IL-6 *in vitro* would affect the expression of *Ntn-1*, *Dcc*, *Shh* and/or *Gli1*.
- **Aim 2**: To investigate whether treatment of mouse E17.5 arcuate nucleus with IL-6 *in vitro* would affect the expression of *Ntn-1*, *Dcc*, *Shh* and/or *Gli1*.
- **Aim 3**: To investigate whether expression changes seen in the arcuate nucleus in response to IL-6 could be replicated *in vivo* using a mouse model of maternal obesity.
Chapter 2: Methods
2.1 High Fat (HF) Mouse Model
All animal procedures performed in this study were approved by the University of Otago Animal Ethics Committee. Female C57BL/6 mice were obtained at eight weeks of age and housed in cohorts of three per cage under standard conditions (25°C, 12hr light-dark cycle, and free access to food and water). Mice were weighed on arrival and randomly assigned to receive either a standard control diet (10% kcals as fat, D12450B, Research Diets, New Brunswick, NJ, USA) or a high fat diet (HF diet, 45% kcals as fat, D12451, Research Diets, New Brunswick, NJ, USA). To keep study sizes manageable a new cohort of three mice per diet type was added every month. Twelve mice from each group were then weighed weekly until the high fat diet group became significantly heavier than the control diet group (Student’s t-test).

2.2 Matings and tissue collection
For in vitro IL-6 experiments chow-fed female C57BL/6 mice were time mated with a chow fed male. Presence of a vaginal plug marked that day as embryonic day 0.5 (E0.5). For in vivo experiments, once the HF diet cohort became significantly heavier than controls, both control and HF fed animals were paired with a chow fed male of normal weight until presence of a vaginal plug was detected. Males were exposed to control or HF diet for no longer than three days while in mating pairs. All adult female mice were killed at E17.5 of pregnancy via cervical dislocation. Embryos were dissected out of the uterus and transferred into either ice-cold artificial cerebral spinal fluid (ACSF) or PBS for in vitro and in vivo experiments, respectively. The head to rump length was measured to confirm embryonic age. Whole heads were removed using fine forceps and the top of the skull dissected off. The heads were then suspended in blocks of 4% agarose (low gelling temperature, Sigma-Aldrich) for stability during sectioning. The proceeding protocol was slightly different for in vitro (section 2.3.1) and in vivo (section 2.3.2) experiments.
2.3.1 *In vitro* IL-6 experiments
Agarose stabilised heads were sectioned using a vibratome (Leica VT2000S) while being completely submerged in ice cold ACSF being actively bubbled with carbogen gas. The agarose block was positioned so the head was nose down, and was sectioned coronally in a caudal to rostral direction, with the blade entering the dorsal surface first. Sections were cut at a thickness of 300 µm, with a vibration amplitude of 1.75 mm, and a speed of 0.6 mm/sec. Sections that contained either arcuate nucleus or hippocampus were transferred into ice cold ACSF being actively bubbled with carbogen gas. Tissue sectioning was completed within 2 hours of the mouse being killed to ensure all tissue remained metabolically active. Sections were transferred into ACSF, pre-warmed to 37°C, and containing either 100 ng/ml interleukin-6 (Human IL-6, Peprotech, Inc.) or no IL-6 vehicle. Sections were incubated at 37°C for one hour. Sections were then microdissected using fine forceps that had been treated with *RNase Away* (Invitrogen) (see figure 2.1). Pieces of arcuate nucleus and hippocampus, from both IL-6 treated and control sections, were pooled in RNase-free microcentrifuge tubes on ice. This experiment was repeated multiple times with embryos from separate pregnancies.

2.3.2 *In vivo* MHF experiments
The heads were sectioned using a vibratome (Leica VT2000S) while being completely submerged in ice cold PBS. Sections were cut as described for our *in vitro* experiments (see section 2.3). Sections that contained either arcuate nucleus or hippocampus were transferred into ice cold PBS and then microdissected using fine forceps which had been treated with *RNase Away* (Invitrogen) (see figure 2.1). All tissue from embryos in the same pregnancy were pooled according to brain area (i.e. arcuate nucleus or hippocampus) and placed in RNase free microcentrifuge tubes on ice. This experiment was repeated multiple times with embryos from separate MHF and control pregnancies.
2.3 RNA extraction and cDNA conversion

RNA was extracted using the Qiagen RNeasy Mini Kit. Tissue was lysed by submersion in the kit lysis buffer, containing 10 μl/ml β-mercaptoethanol, and vigorous vortexing. RNA was extracted and purified with the use of mini spin columns following the kit protocol for extracting RNA from animal tissues. RNA was eluted in 30 μl RNase free H₂O. RNA concentration was quantified using both a spectrophotometer (Nanodrop 2000, Thermo-Scientific) and a fluorometer (Qubit 2.0, Life Technologies). RNA was DNase treated using the Ambion DNA-free kit and following the manufacturer’s protocol. cDNA was created using the Invitrogen’s Supercr ipt III First Strand Synthesis System, following the manufacturer’s protocol and using both random hexamers and OligodTs as primers, and 7 μl of sample RNA. A no reverse transcriptase (no RT) reaction was also run alongside the experimental samples to be sure no genomic DNA or RNA was being carried over and affecting later qPCR results.
2.4 Quantitative PCR (qPCR)

2.4.1 Primer design

Primers were designed for the genes *Ntn1* and *Dcc* (see table 2.1). Design was undertaken using NCBI’s primerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Rozen and Skaletsky, 2000) function to find primer sequences that best fit the following criteria:

- Primer sequence or amplified product fall over intron/exon boundary
- Product size: 80 - 120 bp
- Primer size: 18 - 24 bp
- Primer T<sub>m</sub>: 55 - 65°C
- Primer GC content: min 40% max 60%

Primer pairs were found in the literature for the genes *Shh* and *Gli1*, and a selection of control genes - *Pgk1*, *ActB* and *B2m* (see table 2.1). Gene sequence on Ensembl mouse genome browser (http://www.ensembl.org/Mus_musculus/Info/Index) was used to check candidate primer pairs did not contain known SNP sites, and the Integrated DNA Technologies oligoanalyser (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer) was used to identify primers with low likelihood of forming dimers or hairpin loops.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Amplicon (bp)</th>
<th>Efficiency</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ntn1</td>
<td>F-ACTGCAAGGAGGGCTTCTACCGAGR-CTGGGTGGCAATCACAGGCTTTTG</td>
<td>81</td>
<td>99%</td>
<td>Self designed</td>
</tr>
<tr>
<td>Dcc</td>
<td>F-GCTTTTGTCAGCCAGGACCCACR-ACAGACACGGGAAGCACAAGG</td>
<td>102</td>
<td>100%</td>
<td>Self designed</td>
</tr>
<tr>
<td>Shh</td>
<td>F-CCTTTAGCCTACAAAGCAGTTTATTCCR-GTAATTGGGGGTGAGTCTTAAATC</td>
<td>113</td>
<td>91.7%</td>
<td>Wang et al (2007)</td>
</tr>
<tr>
<td>Gli1</td>
<td>F-TCCACACGCCCTCTAGTG R-TGGCAACATTTTCGCTTGATG</td>
<td>75</td>
<td>83.33%</td>
<td>Wang et al (2007)</td>
</tr>
<tr>
<td>ActB</td>
<td>F-TGTTACCAACTGGGACGACA R-GGGGTGGTTGAAGGTTCTCATAA</td>
<td>153</td>
<td>95%</td>
<td>Larriveé et al (2007)</td>
</tr>
</tbody>
</table>

*Table 2.1: Sybr green primer sequences (5’-3’)*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon (bp)</th>
<th>Fluorescent label</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>ActB</td>
<td>115</td>
<td>VIC</td>
<td>Mmoo607939_s1</td>
</tr>
<tr>
<td>Shh</td>
<td>62</td>
<td>FAM</td>
<td>Mm00436528_m1</td>
</tr>
<tr>
<td>Gli1</td>
<td>68</td>
<td>FAM</td>
<td>Mm004944645_m1</td>
</tr>
</tbody>
</table>

*Table 2.2: TaqMan gene expression information. All assays purchased from Applied Biosystems*
2.4.2 Primer optimisation

A range of primer concentrations (50 nM, 300 nM, 500 nM, 900 nM) were tested on a positive control sample (E12.5 spinal cord cDNA, which has been previously described to express the genes of interest (Bai et al., 2004; Lu et al., 2004; Matise et al., 1999)). The lowest primer concentration with the lowest crossing values was chosen for subsequent experiments. Primer efficiency was tested on four 10-fold dilutions of positive control cDNA (i.e. 470 ng/µl (undiluted), 47 ng/µl, 4.7 ng/µl, 0.47 ng/µl, 0.047 ng/µl). The crossing values were then plotted on a standard curve and the reaction efficiency calculated using the equation:

\[
\text{Efficiency} = 10^{((-1/slope) - 1)}
\]

Using this equation a primer efficiency of 100% indicates the primers amplify the target cDNA by 2-fold each cycle. Values below or above this value indicate poorly designed primers, pipetting error and/or co-amplification of a non-specific product such as primer dimer. All primers used in subsequent experiments fell within the accepted range of efficiency (80-110%) (Schmittgen and Livak, 2008). Melt curves were analysed for each primer set, with a single peak indicating the generation of a single specific product. Additionally, PCR products for each primer set were run on a 1.5% agarose gel. This was then visualised under UV light to check for non-specific products and primer dimers. Taqman gene expression assays (Applied Biosystems) were initially used for Shh and Gli1 with the control gene ActB (see table 2.2). These assays came pre-optimised, so no further optimisation was needed.

2.4.3 qPCR protocol

All qPCR was carried out with the LightCycler 480 Real Time PCR System (Roche) using white 96 well plates. For genes using the TaqMan system (Shh, Gli1, ActB) each individual reaction was run in triplicate and contained: 1 µl 20x TaqMan gene expression assay (Applied Biosystems), 10 µl 2x qPCR mastermix (Applied Biosystems), 7 µl H2O (Molecular grade), 2 µl cDNA. These reactions were run on the LightCycler using the following protocol: 95°C for 10 minutes (pre incubation); 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 1 second (amplification); 40°C for 10 seconds (cooling). For genes using the SybrGreen system (Ntn1, Dcc, Shh, Gli1, ActB, B2m, Pgk1) each individual reaction was run in triplicate and contained: 10 µl 2x SybrGreen mastermix (Applied Biosystems), 2 µl forward
and reverse mixed primer, 6 µl H₂O (Molecular grade), 2 µl cDNA. These reactions were run on the LightCycler using the following protocol: 95°C for 10 minutes (pre incubation); 45 cycles of 95°C for 10 seconds, 60°C for 1 minute and 72°C for 30 seconds (amplification); 95°C for 1 second, 50°C for 1 minute (melting curve); 40°C for 30 seconds (cooling). cDNA sample concentrations were standardised via dilution in fresh MilliQ so that equal ng amounts of experimental and control cDNA (i.e. HF vs control fed, or IL-6 treated vs untreated) were used. All qPCR runs also included both no reverse transcriptase and no cDNA negative controls.

2.4.4 qPCR analysis
qPCR data was initially analysed using the LightCycler 480 software (version 1.5). This involved using the second derivative maximum method to generate a list of crossing values (described in Wellinghausen et al. (2001)). A Student’s t-test was carried out on the crossing values of the reference gene to ensure this gene was not changing significantly between the control and experimental groups. The 2^ΔΔCT relative quantification method (Schmittgen and Livak, 2008) was then used to calculate fold change gene expression in the control and experimental groups. Student’s t-tests were performed to determine significance in gene expression change between the control and experimental group.
Chapter 3: Results
3.1 Optimisation of qPCR

This project was the first time qPCR had been carried out on embryonic tissue in our lab. Due to this, and the lack of literature surrounding appropriate control genes for embryonic arcuate nucleus and hippocampus, a portion of this project was dedicated to optimising a qPCR protocol that worked best for the subsequent experiments in this project.

Firstly, we needed to decide whether the TaqMan qPCR system, which is preoptimised and uses a sequence specific fluorescently labelled probe to measure cDNA amplification; or the SybrGreen qPCR system, which has to be optimised and relies on a fluorophore which binds double stranded DNA to measure amplification was more appropriate. We tested these systems using embryonic hippocampus cDNA from the *in vitro* IL-6 experiments and the genes *Shh* and *Gli1*. When average crossing values were calculated across multiple experiments it was found that those obtained using the TaqMan system were routinely >30, where as SybrGreen values were much lower, when the same amount of sample was run (table 3.1). This meant that for the TaqMan system the number of cycles it took for fluorescence to reach the threshold levels was within 10-15 cycles from the end of the qPCR run, making these results less reliable than those obtained using the SybrGreen system. As the SybrGreen primers showed no signs of co-amplification of nonspecific product (figure 3.1) which could also cause decreased crossing values, it was concluded that the SybrGreen primers were more efficient than the TaqMan system.

Control genes also needed to be found that would remain stable between treatment groups in the E17.5 mouse hippocampus and arcuate nucleus. I began by using *ActB* and *B2m* as control genes because these were widely used in the literature, and had been used on adult rat brain tissue previously in our lab. However, I had many problems with using these genes as controls, as I found that only very occasionally would they stay stable between treatment groups (i.e. IL-6 treated vs. control or MHF vs. control) in either the hippocampus or arcuate nucleus (tables 3.2 and 3.3). Very few qPCR studies have been carried out using embryonic hippocampus or arcuate nucleus so it was hard to find good candidates in the literature. However one paper rated the stability of several qPCR genes in mouse whole embryo and neural stem
cells (Willems et al., 2006). Here, *Pgk1* was ranked highly. I found that *Pgk1* worked well as a control gene for embryonic hippocampus, although I continued to have trouble with it in the arcuate nucleus (table 3.4). This was because the embryonic arcuate nucleus is much smaller than the hippocampus and as a result yielded much less RNA. The small yield of RNA meant that spectrophotometer readings of RNA concentration were inaccurate, and in initial experiments resulted in the amount of cDNA being put into the qPCR reaction not being standardised across treatment groups. This problem was solved by using a fluorometer as well as a spectrophotometer to quantify RNA concentration, so that in the data reported here, all input cDNA amounts were equivalent.

Unfortunately, all of the primer optimisation had to be done using experimental tissue, and even once the protocol was optimised sometimes the concentrations of arcuate nucleus cDNA would still be variable and so could not be used in subsequent analyses. This severely impacted on the amount of time and animals I had available to me. Therefore, sample sizes in some of my experiments are lower than desirable.
Table 3.1: Crossing values are lower using the SybrGreen qPCR system.
Average crossing values for three different samples of E17.5 hippocampus cDNA, each using the TaqMan qPCR system and the SybrGreen system. Equal amounts of cDNA were used for each system.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sample number</th>
<th>TaqMan crossing value</th>
<th>SybrGreen crossing value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shh</td>
<td>1</td>
<td>35.6</td>
<td>26.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>30.1</td>
<td>26.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>34.8</td>
<td>27.2</td>
</tr>
<tr>
<td>Gli1</td>
<td>1</td>
<td>37.3</td>
<td>26.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>30.6</td>
<td>27.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>32.9</td>
<td>27.6</td>
</tr>
</tbody>
</table>

Figure 3.1: Sybr Green primers amplify their specific target sequence only.
Electrophoresis gel of PCR products generated using SybrGreen primers and positive control cDNA (E12.5 spinal cord). Lane 2: Ntn1, Lane 4: Dcc, Lane 6: Shh, Lane 8: Gli1, Lane 10: Pkg1. Odd numbered lanes contain negative control reactions.
Table 3.2: Crossing values using ActB control gene vary significantly between control and IL-6 samples. Average crossing values for three different samples of E17.5 hippocampus and arcuate nucleus cDNA. p values were obtained using a Student’s t-test.

<table>
<thead>
<tr>
<th>ActB</th>
<th>Sample #</th>
<th>Control crossing value</th>
<th>IL-6 treated crossing value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus</td>
<td>1</td>
<td>15.6</td>
<td>16.1</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.7</td>
<td>14.4</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.2</td>
<td>16.4</td>
<td>Not significant</td>
</tr>
<tr>
<td>Arcuate nucleus</td>
<td>1</td>
<td>17.1</td>
<td>16.8</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17.3</td>
<td>15.6</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>18.8</td>
<td>16.0</td>
<td>&lt;0.00005</td>
</tr>
</tbody>
</table>

Table 3.3: Crossing values using B2m control gene vary significantly between control and IL-6 samples. Average crossing values for three different samples of E17.5 hippocampus and arcuate nucleus cDNA. p values were obtained using a Student’s t-test.

<table>
<thead>
<tr>
<th>B2M</th>
<th>Sample #</th>
<th>Control crossing value</th>
<th>IL-6 treated crossing value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus</td>
<td>1</td>
<td>19.6</td>
<td>19.8</td>
<td>Not significant</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20.9</td>
<td>20.7</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>22.0</td>
<td>22.7</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Arcuate nucleus</td>
<td>1</td>
<td>23.7</td>
<td>24.1</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>22.6</td>
<td>21.2</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>21.9</td>
<td>21.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Sample #</td>
<td>Control crossing value</td>
<td>IL-6 treated crossing value</td>
<td>p value</td>
</tr>
<tr>
<td>------------------</td>
<td>----------</td>
<td>------------------------</td>
<td>-----------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>Hippocampus</strong></td>
<td>1</td>
<td>19.9</td>
<td>19.7</td>
<td>Not significant</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>19.7</td>
<td>19.5</td>
<td>Not significant</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>19.2</td>
<td>19.3</td>
<td>Not significant</td>
</tr>
<tr>
<td></td>
<td><strong>Arcuate nucleus</strong></td>
<td><strong>Sample #</strong></td>
<td><strong>Control crossing value</strong></td>
<td><strong>IL-6 treated crossing value</strong></td>
</tr>
<tr>
<td><strong>Arcuate nucleus</strong></td>
<td>1</td>
<td>21.2</td>
<td>21.0</td>
<td>Not significant</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>22.2</td>
<td>22.9</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20.0</td>
<td>20.0</td>
<td>Not significant</td>
</tr>
</tbody>
</table>

**Table 3.4: Crossing values using Pgk1 control gene remain stable between control and IL-6 samples.** Average crossing values for three different samples of E17.5 hippocampus and arcuate nucleus cDNA. Significant change in arcuate nucleus sample 2 was due to inaccurate concentration reading on the spectrophotometer and was resolved by using a fluorometer (see text). p values were obtained using a Student’s t-test.
3.2 *In vitro* IL-6 treatment results in gene expression changes in the fetal hippocampus

We first set out to investigate the role that IL-6 might play in disrupting the expression of developmental genes during fetal development, and thus predisposing to neurological disorders such as schizophrenia. When E17.5 hippocampus was treated *in vitro* for one hour with IL-6 (100 ng/ml) a down-regulation of *Ntn1* and *Shh* expression was observed. *Ntn1* expression underwent a 0.6 fold decrease following IL-6 treatment when compared to control-treated tissue (p<0.001), while *Shh* expression decreased by an average of 0.5 fold (p<0.05). In contrast, *Gli1* expression increased by an average of 1.75 fold (p<0.05). No significant change in *Dcc* expression was observed. Our results suggest a way by which increased levels of IL-6 during pregnancy could disrupt correct development of the hippocampus and predispose to schizophrenia via modulation of important neurodevelopmental genes. However, these data need to be replicated in a model of maternal inflammation or increased IL-6 levels to show they are biologically relevant *in vivo.*
Figure 3.2: Hippocampal gene expression changes in response to in vitro IL-6 treatment. Bar graphs show changes in expression of (A) Ntn1 (n=5 pregnancies), (B) Shh (n=4 pregnancies), (C) Gli1 (n=5 pregnancies) and (D) Dcc (n=6 pregnancies) between control (blue bar) and IL-6 treated (grey bar) embryonic hippocampus. Ntn1 and Shh expression is significantly decreased, and Gli1 expression significantly increased in response to IL-6 treatment. Data represented as the mean ± S.E.M. *=p<0.05, ***=p<0.001 using a Student’s t-test.
3.3 In vitro IL-6 treatment results in gene expression changes in the fetal arcuate nucleus

Next we wanted to investigate how the increased IL-6 levels present in maternal obesity might affect developmental gene expression in the E17.5 arcuate nucleus. One hour in vitro treatment with IL-6 (100 ng/ml) resulted in an up regulation of Dcc, a Ntn1 receptor, and Gli1, a transcription factor within in the Shh signalling pathway. Dcc expression increased an average of two fold (p<0.05) over control. Similarly, Gli1 expression increased an average of 2.5 fold (p<0.05). In the arcuate nucleus Ntn1 expression showed a trend of a slight increase, although this was not significant. Lastly, the IL-6 mediated change to Shh expression in the arcuate nucleus was variable. Expression changes ranged from a 0.35 fold decrease in expression to a 3.2 fold increase. These changes were not significant.

As was the case with the hippocampus, our results show that IL-6 is able to modulate key neurodevelopmental genes, thus providing a possible way in which prenatal IL-6 exposure could affect arcuate nucleus development. While we did not see a significant change in expression of Ntn1 or Shh we did confirm their expression in the E17.5 arcuate nucleus for the first time. Additional experiments might look at the expression of these two guidance molecules outside the arcuate nucleus with a focus on the path axons travel from the arcuate nucleus to their targets in other brain regions. However, examination of these axon tracts would require microdissection of very small regions of tissue and therefore was outside the technical boundaries of the current project.
Figure 3.3: Arcuate nucleus gene expression changes in response to in vitro IL-6 treatment. Bar graphs show changes in expression of (A) Dcc, (B) Gli1, (C) Ntn1 and (D) Shh between control (blue bar) and IL-6 treated (grey bar) embryonic arcuate nucleus. Dcc and Gli1 expression is significantly increased in the IL-6 treated group. Data represented as the mean ± S.E.M. *=p<0.05 using a Student’s t-test. n=4 pregnancies.
3.4 Establishment of a high fat diet mouse model

As part of this project I established a maternal high fat mouse model both for the experiments described here, and for future experiments within our lab. To keep the number of mice I was studying manageable, I kept them in cohorts of three mice in each group (MHF and control diet), introducing a new cohort each month. However due to this small sample size the mice took more than 12 weeks to become significantly heavier than controls, thus delaying experiments that required the use of MHF embryos, and limiting the number of embryos available. This problem was complicated by the variation in the literature for the amount of time it took for C57BL/6 females to become significantly heavier than controls (Grove et al., 2010; Katagiri et al., 2007; Thakker et al., 2006; Tortoriello et al., 2004). However, once data from all cohorts established throughout the year were combined we can be confident that as little as 1-3 weeks of HF feeding is sufficient for most mice to become significantly heavier than controls (p=0.05, figure 3.4). From week 8 onwards weight gain became more stable and significance between the two groups remained at p<0.01, dropping to p<0.001 from week 11. These results demonstrate the successful establishment of a diet-induced obesity in our HF diet mouse model.

We used this model to generate offspring in order to investigate the effects of maternal high fat (MHF) diet on developmental gene expression in the embryonic arcuate nucleus. Thus, after 12 weeks five age-matched female mice were selected for mating from each of the HF diet and control diet cohorts. Figure 3.5 displays their average weights. The HF diet group were significantly heavier than the control diet group at time of mating (p<0.01).

The apparent extended amount of time it took for the HF diet mice to become significantly heavier than controls also impacted on the validation of our MHF model. Ideally I would have liked to validate the MHF model by studying the weight of their offspring to show they are more predisposed to obesity than controls, as has been shown previously (Chang et al., 2008; Grattan, 2008; Howie et al., 2009). Unfortunately, we encountered problems when trying to breed both the MHF mice and their age matched controls, as they would abandon and cannibalise their pups, and whole litters would subsequently die very soon after birth. Younger C57BL/6 females being used in the lab for unrelated experiments were not experiencing these breeding
problems. Additionally advanced maternal age in mice has been linked to a higher pup mortality and incidence of pup cannibalism by the mother (Tarin et al., 2004). Therefore we concluded that it was the advanced age of first mating (~5 months in the HF and control mice) that was causing these problems
Figure 3.4: HF diet-fed mice gain significantly more weight than controls. Line graph showing mean weight of mice fed HF diet (grey diamonds) or control diet (blue squares) plotted against amount of time on diets (n=12/group). Data are represented as the mean ± S.E.M. *=p<0.05, **=p<0.01, ***=p<0.001 using a Students t-test. Mice were 8 weeks old at commencement of diet.
Figure 3.5: HF diet fed mice are significantly heavier than controls at the time of mating. Bar graph showing mean weight of mice fed on control (blue bar) or high fat (grey bar) diet at time of mating (n=5/group). All mice were fed their respective diet for at least 12 weeks. Data represented as the mean ± S.E.M. **=p<0.01 using a Student’s t-test.
3.5 Maternal obesity results in gene expression changes in the fetal arcuate nucleus

Having established the HF diet model, and discovering that IL-6 is able to modulate the expression of several key developmental genes in vitro, the final set of experiments aimed to replicate these results in vivo. MHF fetal arcuate nucleus samples showed similar gene expression changes to those treated with IL-6 in vitro. Dcc expression was 1.8 fold higher in the arcuate nucleus of maternal HF diet embryos (p<0.05). Gli1 was also elevated in the maternal HF diet arcuate nucleus (1.3 fold). However because of the low sample size in this group, statistical testing could not be carried out. Ntn1 and Shh both seemed to be elevated in maternal HF diet arcuate nucleus (an average of 2.9 and 2.7 fold, respectively), however because of the low sample size and variability in the size of the increase these changes did not reach significance. These results are interesting as they mirror the expression changes in vitro by IL-6. This implicates IL-6 levels as an important component of the maternal obesogenic environment causing prenatal programming of offspring obesity.
Figure 3.6: Arcuate nucleus gene expression changes in response to MHF diet mirror in vitro data. Bar graphs show changes in expression of (A) Dcc (n=3 pregnancies/group), (B) Gli1 (n=2 pregnancies/group), (C) Ntn1 (n=3 pregnancies/group), and (D) Shh (n=3 pregnancies/group) between control (blue bar) and MHF diet (grey bar) embryonic arcuate nucleus. Dcc expression is significantly increased in the IL-6 treated group. Data represented as the mean ± S.E.M. *=p<0.05 using a Student’s t-test.
Chapter 4: Discussion
4.1 IL-6 modulates the expression of key developmental genes in the embryonic hippocampus

In this study we have shown for the first time that IL-6 can modulate the expression of key neural developmental genes in the embryonic hippocampus and arcuate nucleus. In the E17.5 hippocampus it was found that in response to in vitro IL-6, the expression of the developmental genes Ntn1 and Shh was significantly down regulated, whereas expression of the transcription factor Gli1 was significantly up regulated. No significant change in the expression of the Ntn1 receptor Dcc was observed.

To date very few studies have examined the effect of prenatal inflammation on neural gene expression. Additionally, most research looks at the adult brain, instead of focusing, as we have, on prenatal brain development. One study that did focus on the prenatal period was conducted by Meyer et al. (2008a). They investigated a selection of genes associated with the development of dopamine neurons, one of which was Shh. It was found that after MIA at E9, both E13 and E17 mouse embryos had significantly decreased Shh expression, similar to our findings in the E17.5 hippocampus after IL-6 treatment. Unfortunately this study measured changes in gene expression in the brain as a whole instead of looking at specific regions. Therefore it was unclear if the mice in the Meyer study showed the same region-specific expression changes as we found with in vitro IL-6 treatment. However in the absence of any other in vivo data for the hippocampus, this is encouraging support of our results.

4.1.1 IL-6 in the hippocampus – relevance to schizophrenia

That IL-6 can modulate expression of developmental genes in the hippocampus is an exciting finding on its own. Further investigation into changes in this region will give us more insights into how maternal inflammation predisposes offspring to developing schizophrenia.

*Disrupted in Schizophrenia 1 (DISC1)* is a candidate schizophrenia risk gene associated with a rare familial form of the disorder, and could also be involved as a more subtle risk factor in other cases (Millar et al., 2000; Singh et al., 2011).
Interestingly, two very recent studies looked at the role of DISC1 in the developing nervous system and found that it was very important in brain development. The first linked its function to Wnt signalling and found that certain SNP variants had significant effects on neurodevelopment in mice and zebrafish, specifically by affecting cell migration (Singh et al., 2011). The second study found DISC1 is important in dendritic growth and adult neurogenesis in the hippocampus (Kang et al., 2011).

The information garnered by these studies is helpful in the context of our own results. For example, in the developing nervous system, Wnt signalling and our developmental genes of interest have a large number of roles in common, namely cell proliferation and migration, and axon guidance (Alves dos Santos and Smidt, 2011). Additionally, Shh is heavily involved in hippocampal neurogenesis in the adult brain (Palma et al., 2005). Therefore, in the absence of DISC1 variants, such is the case with the mice we studied or most humans, IL-6 mediated disruptions to Ntn1, Dcc, Shh and/or Gli1 expression could be an alternative mechanism which disturbs normal neurodevelopment, potentially leading to mental illness. Thus, the gene expression changes we see in response to IL-6 could be programming offspring prenatally towards the development of schizophrenia later in postnatal life.

### 4.1.2 Future directions

The *in vitro* IL-6 treatment data we have obtained is an encouraging indication of the effects maternal inflammation may have on gene expression. However, we need to validate these results in an *in vivo* model. It would be interesting to first look at the expression of these genes in the hippocampi of embryos from MIA mice, and mice injected with only IL-6 during pregnancy, to see if they mirror our current results. It would also be interesting to section brains from these embryos and see whether we could identify any morphological changes in the neurons, such as disrupted cell type specification or axon path finding. This could link the gene expression changes we see in these developmental genes to functional effects in brain development.

Lastly, the range of genes we looked at in the study was very limited in size. In the short term we could try looking at expression changes in other developmental genes.
important in hippocampal development or linked to schizophrenia development, such as those in the Wnt signalling pathway. However this candidate gene approach is a slow way to survey genes that may be affected by IL-6, and is limited by our current knowledge of hippocampal development. To solve this we could use RNA sequencing of the transcriptome to compare hippocampal gene expression changes in maternal IL-6 exposed embryos to those from control mothers. This technique essentially sequences all RNA transcripts present in a sample resulting in information about which genes are expressed and at what levels. By looking at which genes are changing in response to cytokines across the whole transcriptome we would cut down the sheer numbers of genes that are involved in hippocampal development, and may even get a better idea about what genes may be involved in the development of schizophrenia.

4.2 In vitro IL-6 treatment and MHF diet induce similar embryonic gene expression changes in the arcuate nucleus

In the E17.5 arcuate nucleus a very different pattern of gene expression change was observed compared with the hippocampus. While Gli1 expression was once again up regulated by in vitro IL-6 treatment, Dcc was up regulated as well. Shh and Ntn1 showed no significant change. Therefore our results indicate that IL-6 is able to modulate the expression of these important developmental genes in both the embryonic arcuate nucleus and hippocampus.

Interestingly, the E17.5 MHF arcuate nucleus showed a similar pattern of expression changes to those that were IL-6 treated: Dcc was up regulated, and Gli1 looked to be up regulated as well, although small sample size meant statistical analysis could not be carried out. Again, Shh and Ntn1 showed no significant change between control and HFD groups. This provides convincing evidence that it is the increased levels of cytokines, in particular IL-6, in MHF mothers that are causing these changes in gene expression.

4.2.1 IL-6 in the arcuate nucleus – relevance to obesity

It has been well established that cytokine levels are elevated in obese pregnant woman, and several lines of evidence support them as likely candidates for involvement in
developmental programming of offspring obesity (see introduction 1.7). However, what has not been well investigated is exactly which cytokines are important, and how they alter fetal neurodevelopment via developmental programming. Our results support a growing body of literature that implicates increased maternal IL-6 levels in development of offspring obesity. For example one study looking at a variety of metabolic data at time of birth found that increased circulating IL-6 levels, as opposed to other cytokines such as TNF-α, was a good marker for increased offspring adiposity (Radaelli et al., 2006). Additionally a study on rats injected with IL-6 throughout their pregnancy found the offspring had increased amounts of adipose tissue (Dahlgren et al., 2006). This study also found that TNF-α did not have the same effect.

Very little work has been done to date looking at the effects of inflammation on the development of either the embryonic or postnatal arcuate nucleus. Very little is known in general about how the arcuate nucleus develops prenatally, and the role of obesity related inflammation, and specifically IL-6 levels, are only just beginning to be studied as a likely link between maternal and offspring obesity. Therefore the data obtained in this study represents the first time IL-6 mediated developmental gene expression changes have been studied in arcuate nucleus. It is also the first time the effects of IL-6 on development have been established in the context of linking maternal obesity, IL-6 and developmental programming.

4.2.2 Future directions
As so little is known about which genes are important in the prenatal development of the arcuate nucleus the candidate gene approach used in this study is not entirely comprehensive. Here, RNA sequencing to identify changes in gene expression across the transcriptome would be very beneficial. This would give us an idea about what is expressed, and therefore might be important during arcuate nucleus development, as well as what is changing in response to MHF exposure.

In the short term we would like to add more animals to arcuate nucleus IL-6 treatment and MHF diet groups. Of course we would also we would also like to further validate our MHF diet mouse model, especially in regards to IL-6 levels. It would be
interesting to know what the circulating levels of IL-6 are in the HF mice, and whether these changes are mirrored in the placenta and fetal brain. These experiments have not yet been carried out because they are technically challenging due to the small size of the fetal mice, restricting how much tissue can be obtained for analysis. However, in humans this is not such a problem, and the presence of increased IL-6 in maternal obesity, in both the mother and placenta is well established (Challier et al., 2008; Schmatz et al., 2010b). Obviously it is impossible to investigate IL-6 levels in human fetal brain, so this information will have to be obtained from an animal model such as ours.

4.3 Causes of IL-6 mediated changes to developmental gene expression

Our results point to two main explanations as to how expression of these genes is being disrupted by IL-6: (1) that increased IL-6 levels are causing dysregulation in the epigenetic mechanisms that strictly control expression of these genes, or (2) that these developmental genes have an additional function in the immune response.

4.3.1 IL-6 upreualation results in epigenetic changes to the regulation of developmental gene expression

Epigenetics describes changes in the expression of genes caused by modifications that do not affect the primary sequence of the DNA itself (Cox and Nathanielsz, 2009). This includes DNA methylation, and histone modifications, but is also sometimes extended to include post-transcriptional regulators such as microRNAs (Egger et al., 2004; Gluckman et al., 2009).

4.3.1.1 DNA methylation

DNA methylation occurs when a cytosine in the DNA sequence which is immediately followed by a guanine (CpG dinucleotide) has a methyl group attached to it (Cox and Nathanielsz, 2009). Genes that have high levels of methylated CpGs, especially in their promoter regions or first introns, are often transcriptionally repressed (Cox and Nathanielsz, 2009). The idea that maternal environment can cause DNA methylation changes in the developing fetus is a new one, but is rapidly gaining support. Some maternal undernutrition and post-natal over nutrition models have provided some
evidence of abnormal methylation of the Pomc promoter, although they were unable to correlate this with a change in gene expression (Plagemann et al., 2009; Stevens et al., 2010).

We have previously found that Ntn1, Dcc, Shh and Gli1 all have large CpG rich areas in their proximal promoter region or first 5 kb of sequence (appendix 2, figures S1 and S2). Changes in methylation in these genes has been well studied in the context of cancer genetics. While this field is unrelated to the current project, information from these studies does show that these genes are able to undergo changes in their methylation status which in turn has profound effects on their expression levels. For example, hypermethylation of the human DCC promoter has been directly linked to decreased expression levels in a variety of cancers (Hibi et al., 2009; Park et al., 2008; Sato et al., 2001). Changes in promoter methylation status and related gene expression changes during cancer have also been linked to SHH (Fu et al., 2010; ten Haaf et al., 2011; Wang et al., 2006).

In the context of this project, one way in which IL-6 may be able to alter the methylation status of these genes in prenatal infection or obesity would be through the alteration of DNA methyl-transferase (Dnmt) expression. There are two types of Dnmt’s: group 1 (Dnmt1) which maintains established methylation patterns during DNA replication, and group 3 (Dnmt3a, Dnmt3b) which are responsible for laying down the correct methylation patterns early in development (Arzate-Mejia et al., 2011). Both these groups are potentially disrupted by increased levels of IL-6 as they are target genes of the transcription factor Stat3 (Snyder et al., 2008). IL-6 has been shown to have the ability to increase expression of Dnmt1 in cell culture (Hodge et al., 2001). It has been shown that increased expression of Dnmt1 can lead to global hypermethylation of the genome (Biniszkiewicz et al., 2002). This could lead to aberrant methylation patterns being created during cell division possibly accounting for the decrease in expression we see in Ntn1 and Shh in the hippocampus.

Additionally, a study conducted by Fatemi et al (2009) looked at postnatal changes in genes expression in the hippocampus after maternal influenza exposure at E16. Interestingly they found at P0 (day of birth) offspring had decreased expression of Dnmt3a. As this methyltransferase is responsible for setting up the correct pattern of
methylation early in development, reduced expression could lead to the genome being under-methylated and increased expression of certain genes. This mechanism could possibly explain the increase in Gli1 expression we see across all experiments, and the increase in Dec expression in the arcuate nucleus.

4.3.1.2 Histone modification
The other main epigenetic mechanism is histone modification. Histones are proteins which help to package DNA into the chromatin structure. This chromatin can be in an open structure (euchromatin), associated with gene transcription or a tightly packed closed structure (heterochromatin), associated with gene repression (Heerwagen et al., 2010). The structure of the chromatin depends on chemical modifications to the histone proteins themselves (Arzate-Mejia et al., 2011). These modifications consist of acetylation, phosphorylation and methylation of amino acid residues (Heerwagen et al., 2010; Lee and Shukla, 2007).

Histone modifications have been associated with signalling pathways that IL-6 is able to activate. For example, activation of the ERK1/2 pathway has been shown to lead to phosphorylation of histone 3 (Lee and Shukla, 2007). Increased activation of JAK has also been associated with disruption of heterochromatin via phosphorylation of histone 3 (Zouein et al., 2011). Additionally, Youn et al. (2007) have shown in the livers of MIA mice that activated Stat3 can bind the promoter regions of IL-6 mediated target genes. There it actively recruits a methyltransferase complex, modulating the methylation of histone 3 and thus inducing gene expression. It is entirely possible that by this mechanism, or one similar, IL-6 is able to modulate gene expression in the brain.

4.3.1.3 Future directions
Several techniques are available for assessing epigenetic modifications. Firstly, DNA methylation can be assessed using bisulfite conversion, a technique that converts all cytosines in a DNA sequence to uracils, except those protected by methylation. Subsequent PCR amplification and then sequencing of the DNA allows location of where methylated cytosines occurred in the original sequence. This can be performed on a small scale, by designing primers and PCR amplifying CpG rich areas of interest,
such as those occurring in the promoter region. This would be a good pilot study for us to assess promoter methylation in the four genes we studied, and see if this correlates with expression changes. On a larger scale, whole genome bisulfite sequencing can be performed. By using this technique to assess changes in methylation across the whole genome we may be able to identity new candidate genes being disrupted by maternal IL-6.

Additionally several techniques are available to assess histone modifications. Traditionally this kind of analysis was carried out using Chromatin Immunoprecipitation (ChIP) specific to a candidate genomic region, such as a promoter. However high-throughput techniques that combine ChIP with microarray technology (ChIP-chip) or with next generation sequencing (ChIP-seq) are rapidly becoming available, and allow whole genome analysis of differential histone modifications and DNA binding (Heerwagen et al., 2010).

4.3.2 Ntn1 and Shh pathways have a dual function in brain development and in the immune response
The genes investigated in this study are well described as having very important roles in the developing nervous system, notably during axon guidance (see introduction). However, like many genes involved in early development they have recently been found to also to be expressed and possess important functions well after neural development is complete. Of particular interest for this project is that they appear to be key players in the immune response.

4.3.2.1 Netrin-1
Ntn1 has recently been described as having potent anti-inflammatory properties, and in mouse and cell culture models of several inflammatory disorders its expression has been found to be repressed (Mirakaj et al., 2011; Mirakaj et al., 2010; Rosenberger et al., 2009). IL-6 on its own appears to be sufficient to induce this repression (Mirakaj et al., 2010), mirroring our observations of IL-6 mediated repression of Ntn1 in the E17.5 hippocampus. The mechanism for this repression was identified when Mirakaj et al. (2011) found a putative NF-κB binding site in the Ntn1 promoter. Further analysis revealed that activated NF-κB could bind this site, and in doing so actively
repress expression from the Ntn1 gene. IL-6 is able to activate NF-κB (Arzate-Mejia et al., 2011; Ma et al., 2010; Wang et al., 2003) and therefore increased levels of IL-6 are able to repress Ntn1 expression. Although IL-6/NF-κB mediated repression of Ntn1 has not yet been studied in the brain, it is not hard to imagine that elevated levels of maternal IL-6 could interfere with the normal Ntn1 expression pattern required for correct axon guidance.

4.3.2.2 Shh
The Shh pathway has also been described as playing a role during the inflammatory response, this time specifically in the brain. Amankulor et al. (2009) created a mouse model of inflammatory brain injury to study gene expression changes related to astrocytes undergoing reactive gliosis. They found that three days after either mechanical or inflammatory brain injury (induced by injecting bacterial LPS into the cortex) Shh expression and Gli1 activation was increased, which lead to the promotion of cell proliferation around the site of injury. The increase in Shh expression is not surprising because previous studies have found that, like with Ntn1, NF-κB is able to bind the Shh promoter and induce its expression (Kasperczyk et al., 2009). Our current results do not support Amankulor et al.’s findings as we found a significant decrease in Shh expression in the hippocampus, and no significant change in the arcuate nucleus (although there was a trend of increase). However we only treated with IL-6 for 1 hour and Amankulor et al. did not find an increase in Shh until three days post injury. Therefore if our tissue was left in culture for a longer period of time we might expect to find an upregulation of Shh. Additionally, depending on the make up of its subunits, NF-κB can also act as a transcriptional repressor (Verma, 2002). Therefore in the context of prenatal IL-6 exposure, as opposed to adult brain injury, it may well be able to repress Shh expression.

4.3.2.3 Gli1
Expression of Gli1 was upregulated in the hippocampus in response to IL-6 treatment, and in the arcuate nucleus in response to both IL-6 treatment and putatively, MHF diet. Recently it was shown that ERK1/2 signalling is able to increase transcription of Gli1 and therefore the expression of downstream target genes (Seto et al., 2009). As IL-6 signalling stimulates this pathway, this is a mechanism by which increased levels
of IL-6 could directly increase Gli1 transcription and induce Shh signalling in the absence of changes to Shh expression itself. Although it remains unclear, this mechanism could be a cellular response to inflammation and tissue damage, promoting repair through cellular proliferation.

Our experiments indicate that it is almost certain that the changes in Ntn1, Shh and Gli1 expression we have found relate to the ability of these genes to be regulated by cytokines, as they all have roles in the immune response. Under ideal circumstances in utero the developing fetal brain would not be exposed to cytokines, and thus the ability of cytokines to modulate the expression of these genes would never be a problem. However, upon pathological exposure of the fetal brain to cytokines, such as in maternal inflammation or obesity, the expression of these genes is altered, interfering with their important prenatal roles in neural developmental such as, axon guidance.

4.3.2.4 Future directions
Firstly it would be interesting to investigate which cell types in the developing hippocampus and arcuate nucleus express the IL-6 receptor. In the CNS, glial cells such as astrocytes and microglia are known to be involved in mediating immune response during brain injury (Ma et al., 2010). If neurons in these areas are IL-6 responsive it is entirely possible that IL-6 is directly affecting neuronal expression of the developmental genes we looked at. Alternatively, if only astrocytes and microglia in these areas are IL-6 responsive it would suggest that IL-6 is somehow affecting neuronal gene expression indirectly via these cells. Additionally, prenatal IL-6 exposure models (both in vitro and in vivo) could be used to see which IL-6 pathway is activated in IL-6 responsive cells of the embryonic hippocampus and arcuate nucleus. Activation of NF-κB and its translocation to the nucleus would be of particular interest as it seems to be able to activate both Ntn1 and Shh expression. However the activation of other IL-6 pathway components, such as Stat3, would also be of interest as they may be able to modulate neurodevelopmental gene expression in ways we are currently unaware of.
4.5 Concluding remarks

The cytokine IL-6 is elevated in the maternal circulation during infection, and as a result of obesity. Its effects on the developing hippocampus and arcuate nucleus may be key in offspring predisposition to schizophrenia and obesity, respectively. However, a mechanism of how it is affecting neurodevelopment has yet to be discovered.

In this current study, we have shown for the first time the ability of the cytokine IL-6 to modulate the expression of four key neurodevelopmental genes (Ntn1, Dcc, Shh and Gli1) in the embryonic hippocampus and arcuate nucleus. In addition, we have obtained similar gene expression changes in the arcuate nucleus of embryos from mothers fed on a high fat diet during pregnancy. This implicates IL-6 as a key cytokine elevated in obese mothers that is able to modulate expression of genes important in the correct development of the arcuate nucleus, a key neural weight regulation area.

Although the scope of this current research was small, it provides good evidence that increased IL-6 during pregnancy can have a profound effect on the expression of neurodevelopmental genes. The nature and time course of prenatal IL-6 exposure is likely to be very important. Acute high level exposure typical of maternal infection may be more predisposing to schizophrenia, where as chronic low levels may be more associated with development of childhood obesity. Of course, with so many genetic and environmental factors interacting it is unlikely to be this simple, and recent evidence suggests predisposition to schizophrenia and obesity is not entirely mutually exclusive (Pacheco-López et al., 2011). Only further research in this area will help us understand exactly how, and the extent to which, the prenatal environment programs offspring to develop these disorders. In the future, this information will enable us to target prevention to the prenatal period.
Appendix 1

Artificial cerebral spinal fluid (ACSF)
0.118M NaCl
0.003M KCl
0.011M D-glucose
0.01M HEPES
0.025M sodium bicarbonate
0.0005M CaCl
0.006M MgCl₂

1X PBS Buffer
pH 7.4
0.075M sodium phosphate monobasic
0.225M di-sodium hydrogen orthophosphate
0.45M NaCl
Appendix 2

Figure S1: The Ntn1 and Dcc genes contain multiple CpG dinucleotide rich areas in the first 5 kb and proximal promoter region. Schematic representation of the location of CpG dinucleotide rich areas (pink boxes) in the 500 bp upstream (5'), and 5kb downstream (3'), of the transcription start site of the (a) Ntn1 and (b) Dcc genes. Blue boxes indicate exons (Dark blue are coding sequence and light blue are untranslated regions). Lines connecting exons indicate introns. Numbers indicate how many CpG dinucleotides are contained in that region. Arrows indicate transcriptional start site.
Figure S2: The Shh and Gli1 genes contain multiple CpG dinucleotide rich areas in the first 5 kb and proximal promoter region. Schematic representation of the location of CpG dinucleotide rich areas (pink boxes) in the 500 bp upstream (5'), and 5 kb downstream (3'), of the transcription start site of the (a) Shh and (b) Gli1 genes. Blue boxes indicate exons (Dark blue are coding sequence and light blue are untranslated regions). Lines connecting exons indicate introns. Numbers indicate how many CpG dinucleotides are contained in that region. Arrows indicate transcriptional start site.
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