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Gestational overgrowth and undergrowth affect neurodevelopment: similarities and differences from behavior to epigenetics

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Abstract

The size of an infant at birth, a measure of gestational growth, has been recognized for many years as a biomarker of future risk of morbidity. Both being born small for gestational age (SGA) and being born large for gestational age (LGA), are associated with increased rates of obesity and metabolic disorder, as well as a number of mental disorders including attention deficit/hyperactivity disorder, autism, anxiety, and depression. The common risks raise the question of what neurobiological mechanisms are altered in SGA and LGA offspring. Here we review recent findings allowing for direct comparison of neurobiological outcomes of SGA and LGA in human and animal models. We also present new data highlighting similarities and differences in behavior and neurobiology in our mouse models of SGA and LGA. Overall, there is significant data to support aberrant epigenetic mechanisms, particularly related to DNA methylation, in the brains of SGA and LGA offspring, leading to disruptions in the cell cycle in development and gene expression in adulthood.

Keywords

intrauterine growth restriction; macrosomia; DNA methylation; dopamine; opioid; mesolimbic

1. Introduction

Gestation has long been recognized as a critical period for programming lifelong alterations in offspring physiology. Because of this, much attention has been paid to factors influencing gestational quality, and potential markers thereof. One of the most basic outcomes that can be assessed at birth is the size of an infant relative to its gestational age. Infants that are in the bottom 10% of weight for their gestational age (small for gestational age, SGA), even if they are born at term, are at significantly increased risk of mortality and morbidity. These risks include obesity and metabolic disorder, as well as a number of mental disorders such as

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attention deficit/hyperactivity disorder (ADHD), schizophrenia, depression, anxiety, autism, and developmental (cognitive) delay (Hack *et al.*, 2004; Monset-Couchard *et al.*, 2004; Dahl *et al.*, 2006; Kirkegaard *et al.*, 2006; Lahti *et al.*, 2006; Aarnoudse-Moens *et al.*, 2009; Heinonen *et al.*, 2010; Morsing *et al.*, 2011; Ribeiro *et al.*, 2011; Van Lieshout & Boyle, 2011a; Galéra *et al.*, 2011; Colman *et al.*, 2012; Moore *et al.*, 2012). For these reasons, SGA, and the risk factors for intrauterine growth restriction (IUGR) that cause SGA, have been the focus of decades of research aimed at increasing positive childhood health outcomes.

However, it is not necessarily the case that bigger is better when it comes to gestation. As worldwide obesity rates have begun to rise, research has increasingly focused on children born large for gestational age (LGA) or macrosomic, and/or born to mothers who experienced excessive gestational weight gain (GWG), a risk factor for LGA. Emerging data suggest that LGA is potentially as detrimental as SGA. Children born at over the 90th percentile for weight or born to mothers experiencing excessive GWG are at increased risk of mortality, obesity, metabolic disorder, ADHD, depression, anxiety, autism, and cognitive delay (Herva *et al.*, 2008; Rodriguez, 2010; Tamashiro & Moran, 2010; Alfaradhi & Ozanne, 2011; Halmøy *et al.*, 2011; Ornoy, 2011; Van Lieshout & Boyle, 2011b; Colman *et al.*, 2012; Moore *et al.*, 2012).

The overlap in the disorders, both physiological and psychological, experienced by SGA and LGA offspring raises important questions about exactly what mechanisms are perturbed in these conditions, and in turn how these mechanisms are apparently similarly provoked by conditions producing grossly disparate early life phenotypes. It is increasingly clear that disordered gestational growth is one sign of substantial alterations in fetal programming, via epigenetic alterations across the developing organism. We have identified the central nervous system as a particular target of these epigenetic alterations in animal models of SGA and LGA pregnancies, accompanied by similar behavioral deficits. This article will first briefly review factors contributing to the development of SGA and LGA. Subsequently, neurobiological and behavioral similarities and differences in animal models of SGA and LGA will be discussed, including previously unpublished findings from our laboratory that shed additional light on the similarities and differences presenting in our animal models of SGA and LGA.

2. Causes of SGA and LGA

Because of the negative impact of SGA and LGA on offspring survival and prognosis, understanding the factors that increase the probability of abnormal gestational growth is an important first step towards intervention, and an important consideration in the design of animal models designed to compare and contrast the two conditions.

SGA is most frequently associated with primary deficits in placental function, and diseases such as preeclampsia that interfere with placental blood flow. SGA is also associated with adverse maternal behaviors or environmental influences that may create a “secondary” placental dysfunction, such as maternal drug use or maternal undernutrition, particularly insufficient protein levels (Resnick *et al.*, 1982; Myatt, 2006; Godfrey *et al.*, 2011; Bukowski *et al.*, 2012). Additionally, certain genetic imprinting disorders are associated with SGA and

severe neurodevelopment problems (Butler, 2002), and there appears to be a complex genetic association as SGA is more common in women who were themselves SGA when born (Sydsjö, 2011).

LGA, though less frequently recognized in the general public as a condition as problematic as SGA, is also associated with a number of overt disorders, emphasizing its relationship with pathology. LGA is associated with its own cluster of genetic imprinting disorders (Butler, 2002), and LGA is more frequently seen in women with hepatitis B infections (Lao *et al.*, 2012). However, the most frequently seen cause of LGA is gestational diabetes and maternal obesity and excessive GWG during pregnancy, which can in part be caused by overconsumption of high-fat, calorie dense foods (Drake & Reynolds, 2010; Heerwagen *et al.*, 2010).

However, two gestational insults, maternal obesity and maternal stress, are actually associated with increased rates of both SGA and LGA in humans and animal models (Howie *et al.*, 2009; Page *et al.*, 2009; Calegare *et al.*, 2010; Heerwagen *et al.*, 2010; Vucetic, Kimmel, *et al.*, 2010; Djelantik *et al.*, 2012; Oyarzo *et al.*, 2012). The bidirectionality of these effects suggests that maternal diet and maternal stress likely have different effects according to dosage, timing, and downstream effects of the maternal insult. For instance, maternal obesity and prepregnancy high-fat intake predisposes towards SGA and preeclampsia, but is also associated with maternal overeating specifically during gestation, which predisposes towards gestational diabetes and LGA (Rajia *et al.*, 2010; Alfaradhi & Ozanne, 2011), as well as to more subtle gestational growth abnormalities (Hammoud, 2012). The literature on maternal stress supports the importance of considering the timing of the stressor, with SGA and LGA both observed in animal models depending on when the maternal stress was administered (Calegare *et al.*, 2010). Placental development and function is likely a crucial factor, as the ability of the placenta to develop and provide adequate blood flow can be significantly impinged by maternal obesity (Enquobahrie *et al.*, 2008; Heerwagen *et al.*, 2010; Mayeur *et al.*, 2010b; Sébert *et al.*, 2010; Ornoy, 2011), and the inability of the placenta to limit fetal exposure to maternal stress hormones is thought to be a key factor in the severity of maternal stress effects (Markham & Koenig, 2011). As the placenta has been identified as an independent source of a number of neurotransmitters and neuromodulators (including serotonin, leptin, and BDNF), even subtle errors in placental development or function are likely to have deleterious effects on the fetal brain (Lappas *et al.*, 2005; Mayeur *et al.*, 2010a; Bonnin *et al.*, 2011; Maymó *et al.*, 2011).

3. Neurobiological consequences

We will not focus on the broad systemic pathologies which have been described in SGA and LGA offspring, as these have been thoroughly reviewed elsewhere (for example see Ornoy, 2011). As a significant amount of research has been directed at central nervous system (CNS) function in SGA and LGA animal models, rather than attempt a thorough review of this rapidly expanding literature, we would like to highlight several research findings which allow for direct comparisons between SGA and LGA, as they impact neurodevelopment and cognitive function.

It is noteworthy that obesity and metabolic disorder are among the original noted pathologies observed in the Dutch hunger winter studies (Ravelli *et al.*, 1999), which introduced the idea of *in utero* programming of chronic disease. There is clearly an important CNS component to these “peripheral” disorders, and numerous studies have focused on the dysregulation of feeding and satiety pathways in SGA and LGA. Multiple nuclei in the hypothalamus contribute to the central regulation of homeostatic food intake, and the development of this region appears to be significantly impacted in animal models of SGA and LGA (Grayson *et al.*, 2010). Both maternal food restriction (a model of SGA) and maternal high fat diet are associated with significantly increased cell proliferation in the early postnatal hypothalamus (Plagemann *et al.*, 2000; Chang *et al.*, 2008). In the case of maternal high fat diet, it was confirmed that this was an increase in neurons which expressed a number of orexigenic peptides (Chang *et al.*, 2008). In the SGA model, however, it was not clear if these cells were neuronal or glial, but cell counts were specifically increased in the paraventricular, arcuate, and ventromedial nuclei, three key regions in regulating food intake (Plagemann *et al.*, 2000). Intriguingly, a recent study examined the effect of insulin and leptin on embryonic hypothalamic progenitor cells, and found that both hormones, but especially leptin, promoted progenitor cell proliferation, but with leptin promoting a neuronal fate while insulin promoted a glial fate (Desai *et al.*, 2011). Disruptions in the relative levels of these hormones, especially leptin, which is produced not only maternally but in the placenta as well, may play a role in the apparent dysregulation of the cell cycle in the hypothalamus. The shift from neurogenesis to gliogenesis during gestation relies on a shift in DNA methylation patterns (Hirabayashi & Gotoh, 2010), suggesting that gestational endocrine disruptions may play a role in programming epigenetic mechanisms impacting the function of the central nervous system into adulthood, as we see in our data (discussed below). In addition to alterations in the cell cycle and cell fate, evidence suggests that both maternal high fat diets and maternal low protein diets (another animal model of SGA) lead to significant upregulation of hypothalamic intracellular signaling in adulthood, particularly in the leptin-responsive PI3K pathway (Page *et al.*, 2009; Orozco-Solís *et al.*, 2010). Taken together, these data suggest that both maternal over- and under-nutrition promote the over-proliferation of cells in the developing hypothalamus, and the dysregulated activation of key nutrient-sensing pathways as a possible consequence of this activation.

As the hypothalamic literature suggests, epigenetic modifications are central to the effects of gestational growth disorders on CNS development as well as adult neuronal function. Epigenetics can be broadly defined as mechanisms which alter the expression or function of genes without altering the underlying DNA sequence, such as DNA methylation and histone modifications (for review see Levenson & Sweatt, 2005). Epigenetic alterations have traditionally been strictly defined as those changes which are capable of being passed to subsequent generations. However, a wealth of evidence supports the idea that in the CNS the very same epigenetic markers are programmed by experience, including gestational experience through events in adulthood, and that these are not necessarily heritable or indeed similar between any two brain regions or even neuronal subtypes (Mikaelsson & Miller, 2011). As mentioned above, epigenetic modifications, particularly DNA methylation changes, in hypothalamic subnuclei appear to be at the root of gestationally programmed

alterations in cell proliferation and adult neuronal function, and as we shall see, have been identified in a number of other brain structures altered by gestational growth disorders.

As noted in the introduction, SGA and LGA children are at increased risk of many mental disorders continuing into adulthood, strongly implying neurobiological alterations beyond the hypothalamus. Indeed, animal models of SGA and LGA[TR1] have identified deficits in learning and memory. Both conditions have been previously found to reduce the amount of operant responding for a food reward (Mitra *et al.*, 2009; Naef *et al.*, 2011; Reyes-Castro *et al.*, 2011; Rodriguez, Bartlett, *et al.*, 2012; Rodriguez, Rodríguez-González, *et al.*, 2012), suggesting possible deficits with motivational machinery as might be suggested by the gene expression differences we have seen (discussed below). Both SGA and LGA offspring also have problems with spatial learning and memory (Wang & Xu, 2007; Ranade *et al.*, 2008; Bilbo & Tsang, 2010; Tozuka *et al.*, 2010). These deficits in learning and memory corroborate findings of deficits in activation, cell proliferation, and cell volume of the hippocampus in both SGA and LGA (Ranade *et al.*, 2008; Coupé *et al.*, 2009; Niculescu & Lupu, 2009; Bilbo & Tsang, 2010). In SGA, dysfunctional intracellular signaling important to memory formation may be altered by alterations in DNA methylation and histone modifications altering the expression of NMDA receptor subunits and intracellular signaling molecules (Schober *et al.*, 2009; Ke *et al.*, 2011), though it is unknown to what extent these findings occur in LGA. Underscoring these deficits are findings of altered brain-derived neurotrophic factor (BDNF) signaling in the brain and placenta in both SGA and LGA pregnancies (Wang & Xu, 2007; Coupé *et al.*, 2009; Mayeur *et al.*, 2010b). BDNF signaling is a crucial mechanism for altering DNA methylation in neurons and this mechanism is heavily implicated in modulating hippocampal learning and memory as well as hypothalamic function (Martinowich *et al.*, 2003; Day & Sweatt, 2011). Furthermore, BDNF function in healthy placenta appears to be fairly stable (Non *et al.*, 2012), suggesting that alterations in placentally-sourced BDNF in gestational growth disorders may be one mechanism by which DNA methylation in the developing CNS is altered.

Many of the CNS disorders that are associated with SGA and LGA involve dysfunction of the dopamine system - which is also implicated in obesity, as both a regulator of “hedonic” feeding, or the rewarding aspects of food consumption, as well as homeostatic food intake, regulating meal number and meal size. Others have found that early life exposures to a high-fat diet or other forms of overnutrition after birth can profoundly alter the function of the dopamine system in the adult brain, as well as the hypothalamus (Naef *et al.*, 2008, 2011; Teegarden *et al.*, 2009; Coupé *et al.*, 2010; Plagemann *et al.*, 2010). Furthermore, insulin and leptin, which have been shown to program the cellular development of the hypothalamus, also have actions in other brain areas, including the ventral tegmental area (VTA), the major source of dopamine in reward signaling to the rest of the brain (Narayanan *et al.*, 2010). We have been interested in probing the function of striatal, limbic, and cortical dopamine systems in mouse models of SGA and LGA, from a molecular to a behavioral level, and found this system rendered vulnerable in both models.

In our laboratory, SGA and LGA are modeled by manipulating the diet fed to mouse dams during conception, gestation and lactation, using a high-fat (60% calories from fat versus 22% calories from fat in controls) diet to induce LGA (Vucetic, Kimmel, *et al.*, 2010), and a

low-protein (8.5% calories from protein versus 18% calories from protein in controls) to induce SGA (Vucetic, Totoki, *et al.*, 2010; Whitaker *et al.*, 2011). Once born under normal conditions, the LGA and SGA animals differ significantly in weight from controls in the expected direction throughout lactation. Animals are weaned onto control diets, at which point LGA animal weights match that of control offspring, while SGA animals catch up very slowly, if at all, to control offspring weights. [TR2]Under standard conditions, these diets do not affect the number of pups/litter in either SGA or LGA conditions compared to controls. However, two recent incidences of unannounced construction near our breeding facility have provided inadvertent evidence of increased vulnerability of our SGA and LGA litters to stress, and anecdotal support for the importance of the timing of gestational insults in offspring outcomes. In one case, brief but substantial construction noise on days 7 and 9 of gestation reduced the average litter size by 24% and 17% in control and SGA litters, respectively. However, in HF-fed dams, this reduction was over twice as large, with a 58% reduction in litter size, suggesting that the high-fat diet, to which the dams had only been exposed for one week, rendered these pregnancies particularly vulnerable to reabsorption due to stress. The loss of male LGA pups to stress was particularly severe (71% reduction in the number of males versus 46% loss of females), while the pups lost due to stress in control and SGA conditions were evenly divided between males and females. In contrast to the effect of stress during early gestation, a separate cohort experienced construction noise only on gestational day 19 (in our mice, term is gestational day 21). This led to the stillbirth of all pups from 4/9 SGA litters, 2/9 LGA litters, and 0/9 control litters, suggesting maternal stress during late gestation is more damaging under conditions leading to SGA. Prior to this construction work, we observed negligible rates of litter reabsorption and no incidence of stillbirths in our colony. Aside from the unfortunate research setback these events caused, these data provide compelling evidence that the dietary conditions of the dams are sufficient to support pregnancy only if no additional homeostatic/allostatic load is placed on the mother, supporting these as models of at-risk pregnancies in human populations (Langley-Evans, 2007; Entringer *et al.*, 2012).

As stated above, the combined morbidities of obesity, mood disorders, and impulse control disorders seen in both SGA and LGA most obviously implicate the dopamine system, a key neurobiological substrate regulating reward, attention, and action selection. Dopamine (DA) released in most brain regions is synthesized in two midbrain structures: the substantia nigra and the ventral tegmental area (VTA). DA sourced from the substantia nigra primarily regulates voluntary movement, whereas the ventral tegmental area is the source of DA involved in the regulation of reward and mood. The rate-limiting enzyme for DA synthesis is tyrosine hydroxylase (TH). VTA neurons extend projections throughout the brain to a network of structures known as the mesocorticolimbic system, where DA is synthesized and stored for release in VTA axon terminals. Once released, DA exerts actions on postsynaptic neurons via a number of g-protein coupled receptors, including the most common receptor D1, which can complex [TR3]with a number of other receptors including DA receptor D2 and the mu-opioid receptor (Juhász *et al.*, 2008). Activation of D1 leads intracellularly to the phosphorylation of DARPP-32, which plays a role in the action of multiple drugs of abuse and is dysregulated in schizophrenia[TR4] (Yger & Girault, 2011). DA released into the synaptic cleft is removed by the action of the DA transporter (DAT) to prevent prolonged

activation of receptors, and various stimulants including cocaine, amphetamine, and methylphenidate act primarily by blocking the action of DAT. Thus, mood and reward mediated by DA tone and function in key mesocorticolimbic structures including the nucleus accumbens (NAc) and the prefrontal cortex (PFC) is a result of a careful balance between DA synthesis, receptor availability, and reuptake.

In our models of SGA and LGA we see significant perturbations in DA function, as measured at the level of gene expression in both conditions (Vucetic, Kimmel, *et al.*, 2010; Vucetic, Totoki, *et al.*, 2010). In common in both conditions is an upregulation of DAT in the VTA, NAc, and PFC, suggesting increased clearance of DA. However, in SGA this is accompanied by increased TH gene expression measured in the VTA (as well as in the NAc and PFC, presumably from VTA terminals), suggesting a net increase in DA release. Indeed, bolstering the gene expression findings, TH immunoreactivity in the VTA and DA release in the PFC are increased in SGA animals. In contrast, LGA animals display no differences in TH gene expression, but show significant downregulation of the D1 and D2 receptors, as well as downregulation of the DA-activity-linked gene DARPP-32, in the NAc and PFC. Thus, the basic mesocorticolimbic circuitry represented by VTA to NAc and PFC connectivity is seriously dysregulated in both SGA and LGA. However, the effect in SGA is to create a net hyperdopaminergic tone, with TH overactivity and (presumably) compensatory increases in DAT, whereas in LGA a reduction in DA receptors and the intracellular effector DARPP-32 suggests an effectively hypodopaminergic state, due not to reduced DA availability but a reduced capacity to respond to DA and increased clearance of DA via DAT.

In the face of opposing effects of SGA and LGA on the expression of various DA-related genes in mesocorticolimbic regions, it might be reasonable to expect that these conditions would result in divergent behavioral deficits. However, at this time there is only limited data to support behavioral differences, with substantial similarities in other behavioral abnormalities. As a first approach, we have explored the response of SGA and LGA animals to natural rewards (sugar and fat), as well as drugs of abuse (cocaine). Basic reward function as measured by sucrose preference is divergent - LGA animals have increased sucrose preference while SGA animals have decreased sucrose preference, findings which may have more to do with alterations in opioid signaling than with dopamine, discussed further below (Vucetic, Kimmel, *et al.*, 2010; Vucetic, Totoki, *et al.*, 2010). Furthermore, this difference in palatable food preference may not extend to a high-fat diet. LGA animals have an overt preference for a high-fat diet, and while diet preference was not measured directly in SGA animals, we have observed that SGA animals placed on an ad-libitum high-fat diet overconsume the diet relative to control animals. However, without further testing it is not possible to say whether high-fat diet consumption in SGA is driven by homeostatic or hedonic processes[TR5]. Next, we have examined responses to cocaine as a means of pharmacologically probing DA function. We have previously shown that SGA animals show cocaine-induced hyperactivity, and here report that cocaine also induces hyperactivity in LGA animals [FIGURE 1]. However, there appear to be differences in the dynamics of the hyperactivity, such that SGA animals show a comparable peak in hyperactivity but delayed recovery, whereas LGA animals have a much greater peak in hyperactivity coupled with a delayed recovery. Increased stimulant hyperactivity has been linked to overexpression of

DAT (Salahpour *et al.*, 2008), which is common[DM6] in both conditions, and is a model for schizophrenia risk (Papaleo *et al.*, 2012), which may explain the delayed recovery to cocaine, but differences in peak response likely rely on the other observed differences in dopamine signaling.

The behaviors described above have relevance to certain common risks that SGA and LGA infants face, particularly obesity, addiction, and ADHD, but do not fully address other complex and debilitating mental disorders they are at risk of, including mood disorders and social disorders such as autism. We have recently tested behaviors designed to address levels of anxiety and social deficits in our models. A frequently-used test of anxiety in mice is the light-dark box, in which increased amount of time spent in the light half of the box is interpreted as reduced anxiety. However, this behavior, like many of the most common tests of anxiety behaviors, is confounded by activity levels. Both SGA and LGA mice in our tests displayed significant hyperactivity in the light-dark box [FIGURE 2]. It appears that the stress of being placed in the novel chamber for the light-dark box is sufficient to induce hyperactivity (as we do not see baseline differences in hyperactivity in either SGA or LGA animals). While this prevents us from interpreting the time spent in the light side of the chamber to directly assess anxiety levels, it does indicate that both groups are hypersensitive to the stress of the novel chamber, providing indirect support for increased anxiety. Interestingly, another group recently reported that offspring of a high-fat diet made a greater number of entries into the open arm of the elevated plus maze and the center of the open field in those tests of anxiety, without spending more time in those regions (Rodriguez, Rodríguez-González, *et al.*, 2012), which would appear to corroborate our findings of stress-induced hyperactivity. We have also conducted social interaction tests, either by interpreting interactions between unfamiliar dyads (in male mice) or in a 3 chamber test (in female mice). In both conditions, both SGA and LGA animals spent significantly less time overall interacting with the conspecific, indicating deficits in sociability that may be due to increased social anxiety [FIGURE 3] (Franková, 1973).

As it appears that behavioral deficits in SGA and LGA animals extend beyond those that can be strictly linked to dopamine dysfunction, it raises questions about what other systems have been perturbed in the brain. Indeed, while DA gene expression was an obvious target for experimentation, it does not preclude dysfunction in other genes. Currently, we have identified opioid system abnormalities in LGA animals, which may promote increased pleasure in consumption of highly palatable food and other addictive substances (Vucetic, Kimmel, *et al.*, 2010). LGA animals display an overexpression of the mu-opioid receptor (MOR) as well as the gene for its ligand, preproenkephalin (PENK) in the PFC and NAc, suggesting possible oversensitivity to the pleasurable aspects of rewards. We recently followed up on these genes in our SGA animals, which appear to have mildly decreased expression of PENK in the PFC and MOR in the NAc [FIGURE 4]. In short, SGA animals have a decrease in the expression of certain opioid genes (PENK and MOR), while LGA animals show overexpression of these same genes, which is the inverse of the pattern of dopaminergic gene expression observed in these animals. In light of the complex relationship between DA and opioids described in the literature[TR7] (Berridge *et al.*, 2010), these data suggest a potential disassociation between hedonics and motivation as a result of SGA and LGA, which are known to be disassociated in addiction mechanisms (Berridge,

2007). For instance, the differences in sucrose preference between SGA and LGA likely relate to differential opioid function, and we are currently testing whether there is a contrasting pattern of motivated seeking of palatable reward mediated by dopamine. The current opioid data underscore the significance of reward circuitry, of importance in the etiology of many mental disorders, as particularly vulnerable to gestational insults.

In our SGA animals, we have identified dysfunctional regulation of the CDKN1C/IGF2 DNA imprinting locus in the brain, which are important for regulating cellular growth and differentiation, and may be involved with the over-proliferation of neurons others have reported as a result of SGA (Vucetic, Totoki, *et al.*, 2010). However, we have not explored these particular genes in LGA offspring, and consequently we do not know if these abnormalities are shared or unique to one gestational growth pattern.

Although thus far we have identified differing patterns of DA gene expression between SGA and LGA animals, there appear to be similarities in the epigenetic mechanisms that produce these alterations. The overexpression of DAT, MOR, and PENK in LGA animals is related to significant hypomethylation at the proximal promoters of each (Vucetic, Kimmel, *et al.*, 2010). Likewise, the overexpression of CDKN1C and IGF2 in SGA mice is associated with significant hypomethylation at the promoter of these genes (Vucetic, Totoki, *et al.*, 2010). While we do not see significant differences in methylation for other genes for which we saw changes in expression, this does not negate the possible contribution of methylation changes at other sites in a promoter, or a complex interaction of methylation at specific regulatory sites, such as the imprinting control region at the CDKN1C/IGF2 locus. However, it is notable that the only significant differences we see are hypomethylation around genes with altered expression, and thus far have not detected any evidence of promoter hypermethylation.

Because of the centrality of epigenetic modifications in gestational programming, our repeated findings of promoter hypomethylation have led us to take a broader view of the source of similarities between SGA and LGA, with a focus on mechanisms of DNA methylation. We previously found that LGA is associated with global DNA hypomethylation across all brain regions queried in adult animals, implying disruptions beyond the several promoter regions previously queried (Vucetic, Kimmel, *et al.*, 2010). Recently, we tested adult SGA samples and found a similar pattern of global DNA hypomethylation in multiple brain regions [FIGURE 5]. These findings are disturbing, given the importance of DNA methylation in regulating not only gene expression, but also parent-of-origin DNA imprinting, fundamental genomic stability, programming cell fate, and permitting other epigenetic mechanisms including DNA hydroxymethylation and histone modifications. And in fact, significant adverse CNS outcomes have been associated with global hypomethylation, including progressive neurodegeneration, altered neuronal gene expression, and severe degeneration of cortical neurons (Hutnick *et al.*, 2009), as well as behavioral deficits (Woods *et al.*, 2012). These results imply an extensive alteration in epigenetic mechanisms in the adult brain as a result of gestational factors, but do not provide clues as to how this takes place or what the true impact may be.

As a first approach, we are currently exploring whether the DNA methylation “machinery” is compromised in the adult brain as a result of SGA and LGA. In the NAc, we see that both conditions result in a significant increase in DNMT1, the maintenance DNA methyltransferase, and MeCP2, a major methylated-DNA binding protein [FIGURE 6]. We are currently exploring how and why global and promoter-specific DNA hypomethylation (and subsequent gene overexpression) can occur in the face of overexpression of DNA methyltransferase which should lead to increased DNA methylation. Nevertheless, these data are tantalizing in their suggestion that fundamental breakdowns in the DNA methylation machinery in the CNS is a key contributor to the similar dysfunctions in psychological function between SGA and LGA.

4. Overview and Discussion

The principal objective of this manuscript is to address similarities and differences between SGA and LGA offspring in light of their increased risk of neurobiological disorders. Overall, in our data we see evidence that the dopamine and opioid systems, two neurotransmitter systems important to reward, are particularly vulnerable in both SGA and LGA offspring. These effects are reflected in both gene expression changes and behavioral abnormalities. However, at least some of these effects emerge from a background of similar perturbations in DNA methylation machinery in SGA and LGA, which may be a particularly important factor in the CNS development and psychological function of these offspring. One of the most interesting implications of this work is that, as predicted by the neurobehavioral disorders suffered by SGA and LGA offspring, the DA system is particularly vulnerable to abnormal gestational growth patterns, in some ways that are common to both conditions (e.g., forebrain DAT expression), and in other ways which appear sensitive to the gestational growth rate (e.g., VTA TH versus forebrain D1/D2 expression). This may be related to the similar levels of cocaine-induced hyperactivity seen in both conditions (regulated by DAT), versus the disparate levels of sucrose preference (which has been linked to dopamine receptor function in reward). It is possible that the specific programming of dopamine alterations by maternal over- or under-nutrition would program not only preference for specific food sources, but behavioral deficits more specific than increased hyperactivity, leading to subtly different psychological phenotypes. We are currently examining operant chamber performance in SGA and LGA mice to determine if this is the case.

However, it is clear that the DA and opioid systems are not the only vulnerable systems, as we and others have observed a variety of abnormalities in multiple brain regions as a result of both conditions. As some behaviors which demonstrate common deficits in SGA and LGA, such as social interaction, are regulated in a polygenic manner, it seems clear that we have only scratched the surface of CNS abnormalities induced by inappropriate gestational growth. It is very likely that these are related to DNA methylation, which is similarly profoundly dysregulated in both conditions. As a key epigenetic system, disruptions in DNA methylation imply wide-scale changes in gene expression and neuronal function across the brain, which may be largely similar between the two conditions, DA and opioid systems aside. Further research will be necessary to discern the mechanism and impact of DNA methylation dysregulation in SGA and LGA brain, a direction we are pursuing.

One interesting and unanswered question is whether the differences in DA-related epigenetic modifications and gene expression are only consequences of gestational programming, or whether these alterations also propagate further epigenetic alterations. Research focusing on the epigenetics of addiction has identified several mechanisms by which altered dopamine signaling by reward consumption modifies histone marks and DNA methylation in mesolimbic regions (for review see Day & Sweatt, 2011). These include increased BDNF signaling, prevention of histone dephosphorylation by DARPP-32 activation, and altered expression of DNMTs by drugs of abuse. One intriguing possibility is that the consumption of natural rewards such as calorie-dense foods may serve to normalize gene expression in SGA and/or LGA animals by counteracting gestationally programmed patterns of gene expression via these mechanisms. DA-mediated alterations of gene expression in reward regions may support a hedonic basis for overnutrition and obesity in SGA and LGA offspring, in addition to the already identified hypothalamic mechanisms.

Though the data we have reviewed here is interesting on a theoretical and mechanistic level, ultimately we are invested in these findings as translational models of gestational growth abnormalities that afflict human pregnancies and offspring. What do our current results have to say about human SGA and LGA pregnancies? First, they indicate that the increased risk for mental disorders suffered by SGA and LGA humans as they grow beyond infancy is likely based on significant neurodevelopmental abnormalities. In the literature presented here there is evidence of deficits in multiple brain areas important for mood, reward, stress, cognition, and emotion, underscoring the need for vigilant attention to the mental health and development of children who have experienced abnormal gestational growth profiles. Second, given the disparate origins of SGA and LGA in humans, it will be interesting and important to discern whether neurobehavioral outcomes of our maternal diet models occur in other animal models of abnormal gestational growth – in short, whether the cause of the growth difference, or the growth difference itself, is of primary importance. On one hand, cognitive outcomes in infants born preterm and SGA are more damaging than prematurity alone (Morsing *et al.*, 2011), even though both are often caused by similar pathologies, such as preeclampsia, indicating that it is the growth disruption itself, rather than the cause, that is paramount. On the other hand, in animal models of gestational growth disorders, poor-quality maternal diets can produce significant offspring pathologies whether or not significant differences in fetal growth were observed in a particular experiment (Langley-Evans, 2007). Regardless, as excessive GWG is an increasing problem in the first world, and insufficient protein availability is a persistent problem globally (including developed nations), our models certainly reflect influences that are common in the human population and may be a significantly underappreciated cause of mental disorder. Continuing research in this field will hopefully uncover new insights as to the epigenetic mechanisms driving CNS- and genome-wide reprogramming in SGA and LGA offspring.

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Abbreviations

ADHD	attention deficit/hyperactivity disorder
BDNF	brain-derived neurotrophic factor
CNS	central nervous system
DA	dopamine
DAT	dopamine transporter
GWG	gestational weight gain
HF	high-fat
IUGR	intrauterine growth restriction
LGA	large for gestational age
LP	low-protein
MOR	mu-opioid receptor
NAc	nucleus accumbens
PENK	preproenkephalin
PFC	prefrontal cortex
PI3K	phosphoinositide 3-kinase
SGA	small for gestational age
TH	tyrosine hydroxylase
VTa	ventral tegmental area

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Highlights

- Offspring born small for gestational age and large for gestational age share common risks for a number of mental disorders
- The developing brain, particularly the hypothalamus and mesocorticolimbic system, is particularly vulnerable to gestational growth disorders
- DNA Methylation processes are implicated from multiple lines of evidence as a common mechanism of neurobiological dysfunction in gestational growth disorders

Figure 1.

LGA offspring demonstrate an exaggerated locomotor response to cocaine. Male mice (n=8/group) were tested for their locomotor response to cocaine using the Comprehensive Lab Animal Monitoring System (Columbus Instruments). For detailed methods, see (Vucetic, Totoki, *et al.*, 2010). Animals were previously acclimated to the cages and were housed in the cages for 3 days: day 1 (undisturbed); day 2 saline injection (11am); day 3, cocaine (11am, 20 mg/kg, IP). There were no differences in locomotor activity either at baseline or in response to saline injection. In response to cocaine, both control and experimental groups of animals had a significant increase in activity, however, LGA offspring had an exaggerated locomotor response. The peak response was nearly twice that of controls, and these animals also took significantly longer to return to baseline levels of activity. (One-way repeated measures ANOVA, significant interaction between group and time, $F(6,84)=2.25$, $*p<.05$)

Figure 2.

Male (left) and female (right) SGA (gray bars) and LGA (black bars) offspring were tested in the light-dark apparatus ($n=9-10/\text{group}$). Data represent the number of transitions between the light and the dark side of the box during the 5-min test period. In both sexes, SGA and LGA offspring make significantly more transitions as compared to control offspring (Student's t -test). * $p<.05$, ** $p<.01$

Figure 3.

Social interaction in SGA (A) and LGA (B) males, and both SGA and LGA females (C). In males, social interaction was evaluated by placing the target mouse in a novel cage with an unfamiliar male mouse. Behaviors were coded for 10 min. SGA animals showed significantly less time in social behaviors, with no difference in non-social behaviors (Student's t-test) $*p<.05$, $n=5-6$ /group. This was primarily accounted for by a decrease in sniffs. Similarly, LGA animals (B) showed a decrease in the total social time and the number of social interactions, with no difference in nonsocial interactions. (Student's t-test) $*p<.05$, $n=5-6$ /group). Females were evaluated using a 3-chambered social choice task (C). For detailed methods, see (Sankoorikal *et al.*, 2006). The data show the difference score, which is the time spent on the social side of the assay (chamber with a mouse in a cylinder) versus time spent on the nonsocial side (chamber with paper in a cylinder). The SGA females show a clear decrease in time spent on the social side when compared to control animals (which show a positive difference score, indicating that they spend more time in the chamber that houses another mouse, Student's t-test, $p<.05$). LGA animals show a similar pattern to the SGA animals, however, this difference is not statistically reliable. $n=6$ /group

Figure 4.

Expression of target genes in SGA mice was determined by quantitative RT-PCR using gene specific Taqman Probes with Taqman gene expression Master Mix (ABI). For detailed methods, see (Vucetic *et al.*, 2012). Changes in gene expression were calculated against an unchanged GAPDH standard. PENK was significantly reduced in the PFC of SGA mice, while MOR was significantly decreased in the NAc of SGA mice. $n=4/\text{group}$, Student's t -test, $*p<.05$, $**p<.01$.

Figure 5.

Global DNA Methylation in SGA offspring. The amount of global, genome-wide DNA methylation was quantified using the Methylamp Global DNA Methylation Quantification Ultra Kit (Epigentek). For detailed methods, see (Vucetic, Kimmel, *et al.*, 2010). SGA offspring showed significantly reduced global methylation in the VTA, NAc, PFC and HYP, as compared to controls. Student's t-test, * $p < .05$, $n = 6$ /group.

Figure 6.

Expression of target genes in SGA and LGA mice was determined by quantitative RT-PCR using gene specific Taqman Probes with Taqman gene expression Master Mix (ABI). For detailed methods, see (Vucetic *et al.*, 2012). Changes in gene expression were calculated against an unchanged GAPDH standard. DNMT1 and MeCP2 were significantly increased in the NAc in both SGA (top) and LGA (bottom) male offspring. Student's t-test, n=5/group, *p<.05.