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Genomic imprinting and neurobehavioral programming by adverse early life environments: evidence from studying *Cdkn1c*

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Abstract
Imprinted genes are subject to epigenetic regulation that leads to monoallelic expression from one parental allele only. Brain expression of the imprinted gene *Cdkn1c* is sensitive to early life adversity, including exposure to maternal low protein diet (LPD) where increased expression of *Cdkn1c* is due to de-repression of the normally silent paternal allele. Maternal LPD also leads to changes in the dopamine system and reward related behaviours in offspring. We have recently demonstrated that these brain and behavioural phenotypes are recapitulated in a transgenic model in which *Cdkn1c* expression alone is increased. Here we summarise these findings and suggest that the loss of imprinting of *Cdkn1c* in the offspring following maternal low protein diet is a key contributor to the associated changes in the dopamine system and behavior reported after early life adversity.

Introduction
An adverse early life environment (*in utero* and/or early post-natal) can impact on the developing offspring, often resulting in a poor health lasting well into later life. Physiologies affected by exposure to such a sub-optimal environment include the cardiovascular, metabolic and immune systems. An optimal early life environment is also critical for neurodevelopmental outcomes [1] and later-life behavioral phenotypes [2]. Many deleterious effects of adverse environment exposure whilst *in utero*, such as malnutrition and exposure to stress, have been demonstrated in large epidemiological studies (e.g. "Dutch Hunger Winter" and "1959-61 Chinese famines"). These include reduced cognitive abilities [3], as well as increased incidence of schizophrenia [4,5], personality disorder [5] and addiction and gambling problems [6]. These human studies are supported, and indeed have been extended, by a large number of animal studies that show clear associations between *in utero* and/or early post-natal exposure to stress, toxins, low protein and high fat diets, and abnormal brain and behavior in the offspring [1].
There is now a growing need to understand the mechanisms that link exposure to an adverse early life environment and the observed changes in brain and behavior. One suggestion is that an adverse early life environment leads to changes in the epigenetic status, and therefore expression potential, of genes that are important during

neurodevelopment. In this respect, imprinted genes have been posited as possible mediators of early life environmental effects. This group of genes are subject to developmentally determined epigenetic regulation which renders one parental allele transcriptionally silent [7]. Although relatively small in number, imprinted genes are developmentally important [8,9] and even subtle increases or decreases in expression can have significant phenotypic consequences for neurodevelopment [10,11]. Furthermore, a change in the imprinting status of imprinted genes is a recognized functional mechanism within the brain, with de-repression of the normally silent alleles of *Dlk1* and *Igf2* playing a role in neurogenesis [12,13]. As a consequence, imprinted genes may be well placed for mediating environmental effects on neurodevelopment via changes in their epigenetic regulation. However, although limited, current data suggests that generally imprinted genes are relatively insensitive to environmental manipulations, at least in the context of *in utero* nutritional programming and gene expression in metabolic tissues [14,15]. Nevertheless, although studies thus far indicate there is not an enhanced effect of early life programming on changes in expression of this class of genes as a whole, some key imprinted genes do appear to be more sensitive to the effects of a suboptimal maternal diet [14,15].

Cdkn1c expression is altered by an adverse early life environment
One such exception is *cyclin dependent kinase inhibitor 1c* (*Cdkn1c* aka *p57Kip2),* an imprinted gene that is normally silenced on the paternal allele [16]. Expression of *Cdkn1c* in the offspring brain is elevated in response to a number of adverse early life events. Specifically, both maternal low protein diet (LPD) [17], and the degree of early post-natal maternal care [18] lead to increased (2-3 fold) *Cdkn1c* expression. Such alterations in gene expression may reflect changes in expression of the normally active allele, or even changes in cellular composition. However, using a novel *Cdkn1c-luciferase* animal model, we demonstrated that increased expressed of *Cdkn1c* in response to maternal LPD was definitively due to de-repression of the normally silent paternal allele, and accompanied by reduced DNA methylation at the *Cdkn1c* promoter (loss-of-imprinting; LOI) indicating an epigenetic response to prenatal adversity [19]. Importantly, aberrant *Cdkn1c* expression was initiated *in utero* and persisted into adulthood.
Suboptimal diet in pregnancy and poor maternal care are both linked to the abnormal development and function of the offspring dopamine system [20-23]. Increased levels of both Tyrosine hydroxylase (Th, the rate-limiting enzyme in dopamine synthesis) and dopamine have been reported, as well as abnormal reward responses, a behavior known to require dopamine [17,18]. *Cdkn1c* is important in neurogenesis, migration and morphology of the developing nervous system [24-26]. Of particular relevance here is the fact that *Cdkn1c* cooperates with *Nurr1* to promote the proliferation and differentiation of midbrain dopaminergic neurons [27]. Maternally inherited loss of function of *Cdkn1c* results in reduced numbers of Nurr1-positive and Th-positive cells in the ventral midbrain at embryonic day (E)18.5 [27]. Taken together with the recently reported epigenetic sensitivity of *Cdkn1c* to the early life environment, this suggests the possibility that *Cdkn1c* may

contribute to the programming of the offspring dopamine system by abnormal early life environments. However, although its neurodevelopmental function is consistent with a causal role, it is highly likely that *Cdkn1c* is one of many genes whose expression changes following early life adversity. Consequently, to what extent does this subtle elevation in *Cdkn1c* expression contribute to altered behavior later in life?

We recently addressed this question directly using an established *Cdkn1c* transgenic model (*Cdkn1c*[BACx1]) based on a single copy insertion of bacterial artificial chromosome (BAC) spanning the *Cdkn1c* locus into the mouse genome [28]. In this model, *Cdkn1c* is expressed from the transgene in the developing nervous system with temporal and spatial accuracy [28] at approximately 2-fold the normal level modeling LOI [29]. Neural analysis of these animals revealed increased Th staining intensity in the striatum and ventral tegmental area (VTA), increased whole tissue dopamine levels in the striatum, and an enhanced immediate early gene (IEG) response to the stimulant, amphetamine [30], changes reminiscent of those reported after early life adversity [17,22]. In addition to these neuronal changes, the *Cdkn1c*[BACx1] animals showed changes in a number of behaviors linked to the dopamine system.

Dissociation of liking and wanting

The hyperdopaminergic phenotype predicted a change in reward-related behaviors [30,31]. We assessed the hedonic reaction of *Cdkn1c*[BACx1] mice via examination of the microstructure of their consummatory behavior [32]. Rodents typically produce repeated clusters of licks separated by pauses when consuming liquids. The mean number of licks in a cluster (cluster size) is directly related to the perceived palatability of solution being consumed, independent of the overall amount consumed, and is therefore a measure of "liking". *Cdkn1c*[BACx1] animals displayed a lower lick cluster size relative to their wild-type (WT) littermate controls suggesting a reduced hedonic response [12].

Interestingly, when assessed on a progressive ratio schedule (a test of "wanting"), *Cdkn1c*[BACx1] mice were far more motivated by the food reward (8% sucrose), reaching a higher breakpoint (the maximum ratio reached by an animal within a session indicating the point at which they will no longer work for the reward) than their WT littermate controls [30]. Although a dissociation of this kind has been suggested from separate studies [32], the *Cdkn1c* LOI model is one of only two manipulations to show a double-dissociation between wanting and liking within the same animals. Strikingly, the other example is also a hyperdopaminergic animal, produced by knockdown of the dopamine transporter gene, *Dat* [33].

Behavior in the social group

In addition to showing alteration in hedonism, we found that *Cdkn1c*[BACx1] mice were more likely to win a tube-test encounter with unfamiliar animals than their WT littermates, indicative of altered social dominance behaviors [30]. Social dominance has not been shown to be influenced by an adverse early life environment directly, although rat offspring raised

by high licking and grooming mothers do show reduced social interaction and concomitant increased *Cdkn1c* expression [18]. However, previous work indicates that social dominance is governed, in part, by the dopamine system in rodents [34] and that more dominant animals show increased motivation for reward [35].

The enhanced social dominance shown by *Cdkn1c*[BACx1] mice is also interesting in the context of imprinted function generally. Previous work with another imprinted gene, *Grb10*, demonstrated that mice carrying a paternal knockout (*Grb10*[patKO]) were also more likely to win a tube-test encounter with unfamiliar animals [36], paralleling the findings seen in *Cdkn1c*[BACx1] mice. This is the first explicit demonstration of a convergent role for imprinted genes on a behavioral function, paralleling other functional studies indicating a convergent role for imprinted genes in placental function, energy homeostasis and thermogenesis [37]. Moreover, the direction of effects is apparently opposite for maternal *Cdkn1c* and paternal *Grb10*, as twice as much expression of maternal *Cdkn1c* and loss of paternal *Grb10* both increase wins in this test. This pattern fits with the prevailing theory for the evolution of imprinting, namely intragenomic conflict [38], and suggests that the maternal interest is to increase, whereas the paternal interest is to decrease, social dominance.

However, whilst highlighting a possible conflict of interest over a behavioral phenotype, the use of this one tube test outside of the normal social group has been criticized as being unlikely to reflect actual social dominance differences within the normal social group [39]. To address this, we explored the social dominance behavior of *Cdkn1c*[BACx1] mice more deeply and in the more relevant context of the cage-group. We found that levels of *Cdkn1c* expression had no influence on the social dominance rank within the normal home-cage [40]. Nevertheless, the pattern of data did indicate that over-expression of *Cdkn1c* leads individuals to disrupt the normally stable social hierarchy. We interpret this to be because the *Cdkn1c*[BACx1] mice are more territorial than their wild-type cage-mates, shown in other tests, and may contest the social dominance hierarchy more frequently, which in turn leads to a greater incidence of fighting within the social group [40].

Conclusions

These neural and behavioral data suggest that doubling the expression of *Cdkn1c*, mimicking loss-of-imprinting, leads to a hyper-dopaminergic animal [33,41]. In turn, this leads to whole raft of behavioral abnormalities, impacting on reward and social function. Moreover, the phenotype of this model of *Cdkn1c* LOI recapitulates many of the effects seen in animals where manipulation of the early life environment leads to changes in behavior, the midbrain dopamine circuitry and *Cdkn1c* expression [17,18] (Figure 1). This suggests that, although it is likely to result in a number of gene expression changes, the loss of imprinting of *Cdkn1c* in the offspring following maternal low protein diet is a key contributor to the associated changes in the dopamine system and behavior reported after early life adversity. However, whether this particular gene expression increase is *the* critical change, still remains to be fully established.

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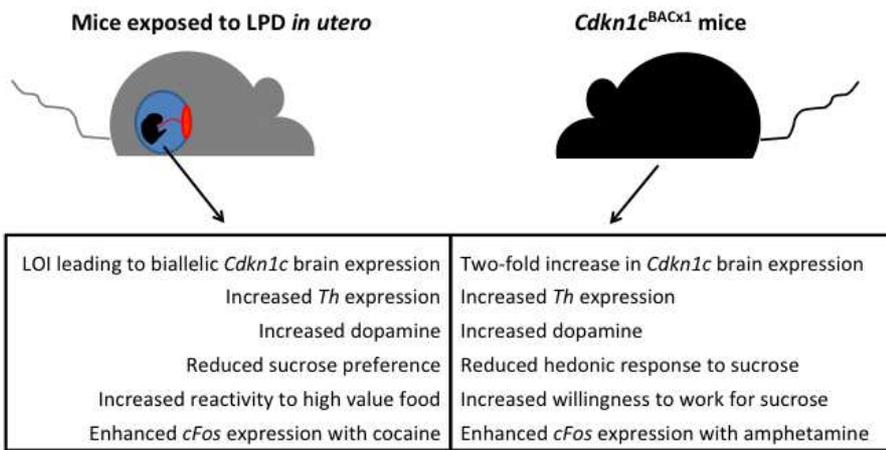
Mice exposed to LPD *in utero*

Cdkn1c^{BACx1} mice

LOI leading to biallelic *Cdkn1c* brain expression	Two-fold increase in *Cdkn1c* brain expression
Increased *Th* expression	Increased *Th* expression
Increased dopamine	Increased dopamine
Reduced sucrose preference	Reduced hedonic response to sucrose
Increased reactivity to high value food	Increased willingness to work for sucrose
Enhanced *cFos* expression with cocaine	Enhanced *cFos* expression with amphetamine

Figure 1 Schematic summarising similar brain and behavioural phenotypes seen mice exposed to maternal LPD and transgenic *Cdkn1c*^{BACx1} mice (LOI = loss of imprinting).