Dopaminergic and behavioral changes in a loss-of-imprinting model of Cdkn1c

Gráinne I. McNamara¹, Brittany A. Davis², Molly Browne², Trevor Humby¹,³, Jeffrey W. Dalley⁴,⁵, Jing Xia⁴, Rosalind M. John² & Anthony R. Isles¹*

¹Behavioural Genetics Group, MRC Centre for Neuropsychiatric Genetics and Genomics, Neuroscience and Mental Health Research Institute, Cardiff University, Cardiff, United Kingdom CF24 4HQ
²School of Biosciences, Cardiff University, Cardiff, United Kingdom, CF10 3AX
³School of Psychology, Cardiff University, Cardiff, United Kingdom CF10 3AT
⁴Departement of Psychology, University of Cambridge, Cambridge, United Kingdom, CB2 3RQ
⁵Department of Psychiatry, University of Cambridge, Cambridge, United Kingdom, CB2 0SZ

*Corresponding author: Email: IslesAR1@cardiff.ac.uk; Tel. +44(0)2920 688467

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Abstract

The imprinted gene *Cdkn1c* is expressed exclusively from the maternally inherited allele as a consequence of epigenetic regulation. *Cdkn1c* exemplifies many of the functional characteristics of imprinted genes, playing a role in fetal growth and placental development. However, *Cdkn1c* also plays an important role in the brain, being key to the appropriate proliferation and differentiation of midbrain dopaminergic neurons. Using a transgenic model (*Cdkn1c*BACx1) with a two-fold elevation in *Cdkn1c* expression that mimics loss-of-imprinting, we show that increased expression of *Cdkn1c* in the brain gives rise to neurobiological and behavioural changes indicative of a functionally altered dopaminergic system. *Cdkn1c*BACx1 mice displayed altered expression of dopamine system related genes, increased tyrosine hydroxylase (Th) staining and increased tissue content of dopamine in the striatum. In addition, *Cdkn1c*BACx1 animals were hypersensitive to amphetamine as revealed by *c-fos* expression in the nucleus accumbens. *Cdkn1c*BACx1 mice had significant changes in behaviours that are dependent on the mesolimbic dopaminergic system. Specifically, increased motivation for palatable food stuffs, as indexed on a progressive ratio task. In addition, *Cdkn1c*BACx1 mice displayed enhanced social dominance. These data demonstrate, for the first time, the consequence of elevated *Cdkn1c* expression on dopamine related behaviours highlighting the importance of correct dosage of this imprinted gene in the brain. This work has significant relevance for deepening our understanding of the epigenetic factors that can shape neurobiology and behaviour.
Introduction

Imprinted genes represent a class of genes that are monoallelically expressed, allelic expression being dependant on the parent of origin, as a result of epigenetic events initiated in the germline (Ferguson-Smith, 2011). This class of genes were identified following on from the observation of the non-equivalence of the parental genomes in androgenetic, gynogenetic and parthenogenetic murine embryos resulting in embryonic fatality (Mcgrath & Solter, 1984, Surani et al., 1984). The function of these genes converge on key aspects of mammalian physiology including placental development and function (Tunster et al., 2013), metabolism (Smith et al., 2006), and behaviour (Davies et al., 2015). Furthermore, imprinted gene function is sensitive to dosage as exemplified by human imprinting disorders that arise from improper imprinted gene expression levels. These include Beckwith–Wiedemann syndrome, Silver–Russell syndrome (SRS) (Demars & Gicquel, 2012), Prader-Willi and Angelman syndromes (Buiting, 2010).

Cyclin dependant kinase inhibitor 1c (Cdkn1c) is a maternally expressed imprinted gene located in the Kcnq1 imprinting locus on mouse distal chromosome 7/human chromosome 11p15. Cdkn1c (aka p57Kip2) is a member of the Cip/Kip family of cyclin dependant kinase inhibitors (CDKi) (Lee et al., 1995, Matsuoka et al., 1995). Expression in the brain peaks embryonically with restricted postnatal and adult expression (Furutachi et al., 2013, Furutachi et al., 2015, Westbury et al., 2001). In the developing nervous system Cdkn1c plays a role in neurogenesis, migration and morphology (Itoh et al., 2007, Joseph et al., 2003, Tury et al., 2011, Ye et al., 2009). Independent of its CDKi function, Cdkn1c cooperates with Nurr1, to promote the proliferation and differentiation of midbrain dopaminergic neurons (Joseph et al., 2003). Maternally inherited loss of function of Cdkn1c results in reduced numbers of Nurr1-positive and Th-positive cells in the ventral midbrain on E18.5 (Joseph et al., 2003). Study of Cdkn1c function post-natally has been limited due to the perinatal lethality of a global knockout (Yan et al., 1997, Zhang et al., 1997). In the brain, experiments utilising a conditional knockout of Cdkn1c have shown that this gene functions to
maintain quiescence of adult neural stem cells (Furutachi et al., 2013, Furutachi et al., 2015).

We have previously demonstrated a role for Cdkn1c in the behavioural symptoms associated with the imprinting disorder, Silver Russell Syndrome (SRS), specifically relating to hedonic responding to a palatable food stuff (Mcnamara et al., 2016). Here we model the consequences of loss-of-imprinting of Cdkn1c using a BAC transgenic mouse model (Cdkn1c^{BACx1}) that resulted in elevated Cdkn1c in a subset of tissues, including in the developing nervous system, at two-fold the endogenous level (Andrews et al., 2007, John et al., 2001).

We report that a double dosage of Cdkn1c results in an altered dopaminergic state in the mesolimbic system, with elevated Th and neurotransmitter levels in the striatum associated with increase Th-positive cell number in the ventral tegmental area (VTA). Corresponding to this, changes in a number of behaviours, specifically motivation for a food reward and social dominance, were observed. These findings lend further support to the importance of genomic imprinting in mediating complex mammalian behaviours and emphasise the significance of Cdkn1c expression for dopaminergic system function.

MATERIALS AND METHODS

Animals

All procedures were conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986, under the remit of Home Office licence number 30/2673 with additional ethical approval at Cardiff University.

The experimental line Cdkn1c^{BACx1} possesses one copy of a bacterial artificial chromosome that spans the Cdkn1c gene and two other genes, Phlda2 and Slc22a18. The reporter line Cdkn1c^{BAC_{Lac}} possesses a modified version of this BAC with a β-galactosidase reporter construct inserted into the Cdkn1c locus, disrupting Cdkn1c expression (John et al., 2001). Cdkn1c^{BAC_{Lac}} functioned as a control for the experimental line to attribute any phenotypes observed specifically to the over expression of Cdkn1c (i.e. present in line Cdkn1c^{BACx1}, absent in line Cdkn1c^{BAC_{Lac}}). Both lines are maintained on a C57BL/6J
background and have been bred onto this background for >8 generations. For embryonic tissue wild-type (WT) C57BL/6J females aged 6 to 8 weeks was paired with a Cdkn1c<sup>BACx1</sup> male. Presence of a vaginal designated embryonic day (E)0.5. On E18.5 embryos were isolated and forebrain was separated from mid- and hindbrain. Tissue was snap frozen and stored at -80°C until processing.

Subjects were male mice. For the social dominance tube test task experiment N=71 animals were used in total: Cdkn1c<sup>BACx1</sup> (n=23) and their WT littermates (n=19); Cdkn1c<sup>BAClacz</sup> (n=14) and their WT littermates (n=15). A subset of these were used for progressive ratio testing: Cdkn1c<sup>BACx1</sup> (n=14) and their WT littermates (n=13); Cdkn1c<sup>BAClacz</sup> (n=17) and their WT littermates (n=9). Animals were between 8-12 weeks old for social dominance testing, and 13 and 15 weeks at the start of progressive ratio testing. Standard laboratory chow was available ad libitum, but just prior to and during the progressive ratio experiment, water was restricted to 2h access per day. This regime maintained the subjects at ~90% of free-feeding body weight. All behavioural testing was performed in the light phase (lights on 07:00, for 12-hours).

**RNA extraction and qPCR**

Tissue for RNA analysis was obtained following cervical dislocation and isolation of the brain. To assess sensitivity to amphetamine an i.p. injection of saline or 0.5 mg/kg D-amphetamine (Tocris Bioscience, Bristol, UK) was administered. Brains were isolated 60 minutes following injection by cervical dislocation and ventral striatum was isolated. Embryonic RNA was extracted using PARIS kit (Life Technologies, UK). Adult RNA was extracted using standard phenol extraction methods and DNase treated using RNAse-free DNase treatment and removal kit (Applied Biosystems, UK) according to the manufacturer’s instructions. Expression was normalised (∆Ct) to the geometric mean Ct value of β<sub>actin</sub> and Hprt as these genes have previously been verified in-house. Statistical analysis was carried out on transformed ∆Ct values.

**Immunohistochemistry and image analysis**

Immunohistochemistry was carried out as previously described (Mcnamara et al., 2016). Briefly, animals were transcardially perfused with 10% formalin fixative
(Sigma-Adrich, UK) and brains removed. Following post-fixing overnight, brains were removed to a solution of PBS containing 30% sucrose. 40 µm sections were obtained on a freezing microtome. Immunostaining was carried out on free-floating sections. For striatal analysis, adjacent sections were incubated in a single primary antibody (anti-NeuN (1:1000) (Abcam, UK) or rabbit polyclonal anti-Th (1:1000) (Abcam, UK)) in 3 % NGS TBS-T overnight at 4°C. For analysis of the VTA a separate cohort of tissue was prepared as above and staining was carried out on every 1 in 6 sections. The VECTASTAIN ABC kit (Vector Labs, Peterborough, UK) and DAB peroxidise substrate kit (Vector Labs, UK) were used as per manufacturer’s instructions. Images were acquired at 1.5X (for Th immunoreactivity) or 5X (for NeuN immunoreactivity) magnification using Leica Olympus DP73. 20X Th cell counts in ventral tegmental area (VTA) and substantia nigra pars compacta (SNc)) were acquired using Olympus BX41 brightfield microscope with slide-scanning adapter. For Th immunoreactivity all sections containing the striatum were imaged. For NeuN 10 sections distributed evenly throughout the striatum were chosen for analysis. A single image was taken of the striatum and another of the adjacent cortex, for each hemisphere, a total of 20 images per animal. All image analysis was carried out using ImageJ. For striatal Th average optical density of the striatum, separated into dorsal and ventral regions, was obtained and the average background staining from the cortex was subtracted to give a value comparable between sections. Cell number (NeuN and Th) was calculated using ImageJ cell counter plugin (NIH ImageJ software version 1.45s; http://rsb.info.nih.gov/ij/) on the 8-bit black and white images. Th cell number in VTA and SNc was counted manually by experimenter blind to genotype.

Whole tissue neurochemistry

Animals were sacrificed by exposure to a rising concentration of carbon dioxide and cervical dislocation. The brains were rapidly removed and the dorsal striatum dissected. Tissue aliquots were derived from both hemispheres and homogenized in 200 µl of 0.2 M perchloric acid by an ultrasonic cell disruptor (Microson, UK). Levels of NA, DA and 5-HT were determined in the supernatant
by reversed-phase, high-performance liquid chromatography, as described previously (Dalley et al., 2002).

**Progressive ratio**

For the duration of testing animals were on a restricted water access schedule, water provided for two hours immediately after testing. Briefly, testing took place in a 9-hole box modified for use in mice. During testing a nose-poke (NP) in the illuminated hole resulted in the presentation of 20 µl of an 8% sucrose reinforce. Collection of this reward initiated a subsequent trial. Continuous reinforcement (CRF - i.e. one nose poke required for reward delivery) was carried out for four days. Following this, a progressive ratio schedule was carried out. Number of nose pokes required ascended linearly every four trials (Ratio 4, FR4) for three sessions, followed every two trials (Ratio 2, FR2) a further three sessions. The maximum number of nose poke an animal was willing to make to receive a reward is deemed break-point (BP), and is an indication of the animal’s motivation to work for a reward. These progressive ratio sessions were followed by four more CRF sessions.

Following this basal analysis, single probe trials at the ratio FR2 progressive ratio schedule were carried out using 2% (w/w) sucrose and calorie free saccharin (0.1% (w/w)). To exclude effects of neophobia on saccharin BP, animals were trained to obtain a reward of 0.1% saccharin in a CRF schedule for two days prior to this a probe trial at FR2.

Two infrared beams at front and rear of arena provided a measure of activity during trials. Each break in the beam was counted and total beam breaks in a trial was analysed.

**Social dominance test**

The tube test was carried out as described in Garfield et al. (Garfield et al., 2011). Briefly, the test apparatus consisted of a 30 cm, transparent tube with a 3.5 cm diameter placed in an opaque arena to obscure view of the environment. Testing was carried out in dimmed light conditions. At the beginning of each trial two animals were introduced into the tube from both ends and released simultaneously. A trial was complete when one animal fully backed out of the
The animal that did not back out was considered the dominant animal of the trial. Each dyadic encounter consisted of one one transgenic and one WT animal, these did not differ in weight by more than 10%. Each transgenic animal had three encounters with three different, unfamiliar, WT animals. The number of encounters won by either transgenic or WT animals was recorded.

**Statistical analysis**

All statistical analysis was carried out using SPSS 20.0 (SPSS, USA). For analysis of *Dat* expression, dopamine concentration in the dorsal striatum and Th cell number in VTA and SNc, Student’s t-tests were carried out with GENOTYPE (WT or transgenic – either *Cdkn1c*<sup>BACx1</sup> or *Cdkn1c*<sup>BACLacZ</sup>) as a grouping variable. For Th expression and IHC analysis a repeated measure ANOVA was carried out with GENOTYPE as a between subject factor and REGION as a within subject factor. For analysis of progressive ratio a series of mixed ANOVAs were carried out on the data with a between subject factor of GENOTYPE and within subjects factors of SESSION or SOLUTION (sucrose or saccharin) where appropriate. Bonferroni post-hoc tests were carried out where appropriate. Activity during progressive ratio testing was analysed using a Student’s t-tests were carried out with GENOTYPE as a grouping variable. Dominance behaviours in the tube test were assessed by a binomial non-parametric test where all encounters were coded ‘0’ for those won by WT or coded ‘1’ for those won by transgenic (either *Cdkn1c*<sup>BACx1</sup> or *Cdkn1c*<sup>BACLacZ</sup>). Expected proportion was 0.5 assuming each encounter was equally likely to be won by either WT or transgenic.

**RESULTS**

**Increased *Cdkn1c* dosage results in a modified basal dopaminergic system in the striatum**

As previously demonstrated (Andrews *et al.*, 2007, John *et al.*, 2001), *Cdkn1c* expression was elevated in the forebrain in *Cdkn1c*<sup>BACx1</sup> animals on embryonic day (E)18.5 (t(5)=−6.9, p=0.001) (Supplementary Material, Figure S1). In adult *Cdkn1c*<sup>BACx1</sup> animals elevated expression of *Cdkn1c* resulted in an increase in Th protein immunoreactivity in the ventral striatum (t(6)=−3.49, p=0.013) but not
the dorsal striatum $t(6)=-1.5$, $p=0.18$) of $Cdkn1c^{\text{BACx1}}$ animals (Figure 1A).

Correspondingly, $Th$ mRNA was increased 1.9 fold in the ventral striatum, though this didn’t reach statistical significance ($t(6)=-1.89$, $p=0.11$). $Th$ mRNA was unchanged in the dorsal striatum ($t(6)=-0.76$, $p=0.48$) (Figure 1B). There was no significant change in the $Dopamine$ transporter ($Dat$) mRNA in the ventral striatum ($t(5)=1.5$, $p=0.19$) of $Cdkn1c^{\text{BACx1}}$ animals compared with WT littermates. However, in the dorsal striatum there was a nine-fold increase in $Dat$ mRNA ($t(5)=4.696$, $p=0.005$) in the dorsal striatum, this was accompanied by a 20% increase in whole tissue level of dopamine in the dorsal striatum of $Cdkn1c^{\text{BACx1}}$ animals (Figure 1D; $t(14)=-2.298$, $p=0.038$), without a change in the metabolite DOPAC ($t(14)=-0.323$, $p=0.75$) or in turnover ($t(14)=0.874$, $p=0.397$, data not shown). Despite the elevated $Th$ protein staining in the ventral striatum, there was no effect of elevated $Cdkn1c$ expression on dopamine, DOPAC or turnover in this region (dopamine: $t(14)=-0.8$, $p=0.93$; DOPAC: $t(14)=-0.63$, $p=0.54$; turnover: $t(14)=0.25$, $p=0.81$) (Figure 1E). Given the cell cycle regulatory function of $Cdkn1c$, an important histological feature to assess was total neuronal cell number. There was no effect of elevated $Cdkn1c$ on striatal ($t(8)=0.23$, $p=0.98$) or surrounding cortical cell number ($t(8)=1.15$, $p=0.28$) as determined by number of NeuN positive cells (Figure 1F).

In all measures, the $Cdkn1c^{\text{BACx1}}$ transgenic control mice showed no phenotypic differences from WT (see Supplementary Material, Figure S2A-E).

$Cdkn1c^{\text{BACx1}}$ mice have an altered neural response to amphetamine

The ventral striatum encompasses the nucleus accumbens, a structure central to reward processing, and receives a direct dopaminergic input from the VTA. To probe the altered dopaminergic phenotype of $Cdkn1c^{\text{BACx1}}$ animals further we assessed the neural responsivity of this region to a single dose of amphetamine by measuring transcript levels of the immediate early gene $cfos$ 60-minutes post-injection. Following a low dose (0.5 mg/kg) injection of amphetamine ANOVA revealed a GENOTYPE*DRUG interaction ($F_{1,17}=8.687$, $p=0.009$), with post-hoc analysis revealing increased $cfos$ expression in the ventral striatum in animals with elevated $Cdkn1c$ (Figure 2A; $F_{1,17}=7.467$, $p=0.014$) but a not in their WT littermates ($F_{1,17}=1.827$, $p=0.194$). This finding implies a greater response
magnitude in cells of $\text{Cdkn1c}^{\text{BACx1}}$ animals or a larger number of cells activated in response to amphetamine stimulation. To address this, Th positive cells in the VTA, the mesolimbic input to the ventral striatum, were quantified. $\text{Cdkn1c}^{\text{BACx1}}$ had significantly more Th-positive cells in the VTA than WT (Figure 2B; $t(8)=2.32$, $p=0.049$). There was no difference in Th-positive cell number in the adjacent SNc (Figure 2B; $t(8)=0.98$, $p=0.37$).

**Elevated $\text{Cdkn1c}$ dosage increases breakpoint on a progressive ratio task**

During CRF trials, where a single nose-poke elicited an 8% sucrose reward, there was no difference in number of trials completed between WT and $\text{Cdkn1c}^{\text{BACx1}}$ animals (Figure 3A; main effect of GENOTYPE: $F_{1,23}=2.51$, $p=0.127$). However, in a progressive ratio schedule, during which the number of nose pokes required to receive a reward ascends within a session, $\text{Cdkn1c}^{\text{BACx1}}$ animals had a 1.5- to 2-fold higher BP than their WT littermates (Figure 3B; $F_{1,23}=17.109$, $p<0.001$). This implies that $\text{Cdkn1c}^{\text{BACx1}}$ will work harder to receive a reward than WT. In addition to a higher BP, $\text{Cdkn1c}^{\text{BACx1}}$ animals had a shorter inter nose-poke interval (Figure 3C; $F_{1,23}=28.56$, $p<0.001$), and were quicker to complete trials (Figure 3D; $F_{1,23}=12.442$, $p=0.002$). Importantly there was no general difference in activity as measured by total beam breaks during the task ($F_{1,23}=0.179$, $p=0.676$) (data not shown). Together these data indicate that two-fold elevated expression of $\text{Cdkn1c}$ increases directedness as well as motivation to obtain a sucrose reward.

In all these measures on the PR task, the $\text{Cdkn1c}^{\text{BAClaZ}}$ transgenic control mice showed no phenotypic differences from WT (Supplementary Material, Figure S3A-D).

**Performance of $\text{Cdkn1c}^{\text{BACx1}}$ mice is driven by motivation to obtain reward**

We explored the motivational phenotype of $\text{Cdkn1c}^{\text{BACx1}}$ mice on the progressive ratio task further by assessing the effect of manipulations of the reinforcer reward at an FR2 schedule. Firstly, we decreased the sucrose solution from 8% to 2%. Animals had equivalent breakpoints for 2% and 8% sucrose (Figure 4; $t(24)=0.585$, $p=0.56$), and animals with elevated $\text{Cdkn1c}$ expression continued to
work harder than WT littermates to receive a 2% sucrose reward, as indexed by a significantly higher BP ($t(23)=-3.334$, $p=0.003$).

To differentiate between the calorific and hedonic rewarding properties of sucrose, the animals’ motivation to work calorie- free, saccharin (0.1% (w/w) saccharin) was assessed in the progressive ratio schedule. Animals were first trained to consume saccharin on a CRF schedule for two days with $Cdkn1c^{BACx1}$ and WT animals completing an average of 67.5 (5.2 SEM) and 76.14 (4.6 SEM) trials respectively. Animals were then moved onto an FR2 schedule. All animals were less motivated to work for saccharin compared to 8% sucrose, as indexed by a decrease in BP (Figure 4; main effect of SOLUTION $F_{1,23}=10.293$, $p=0.004$). Bonferroni post hoc test showed this to be driven primarily by the significant decrease in BP of $Cdkn1c^{BACx1}$ mice ($F_{1,23}=10.578$, $p=0.004$) compared to a non-significant decrease in WT littermates ($F_{1,23}=0.174$, $p=0.174$). Nevertheless, despite this overall decrease, BP remained significantly higher in $Cdkn1c^{BACx1}$ mice than WT even when consuming saccharin (Figure 4; $t(23)=-2.872$, $p=0.009$).

**Elevated $Cdkn1c$ dosage increases social dominance**

Successful performance in a tube test is associated with a dominant status in rodents (Lindzey et al., 1961). When $Cdkn1c^{BACx1}$ males were paired with unfamiliar, weight-matched WT males, the transgenic animals won significantly more encounters (Figure 5; 63.1% vs. 36.9, $\chi^2=4.45$, $p=0.046$). There was no significant difference in the proportion of encounters won by the control $Cdkn1c^{BAC\text{LacZ}}$ males compared to unfamiliar, weight-matched wild-type animals (Figure 5; 46.7% vs 53.3%, $\chi^2=0.2$, $p=0.655$).

**DISCUSSION**

In this study, we examined the consequence on brain and behaviour of an increase in expression of $Cdkn1c$, a maternally expressed imprinted gene critical for early development. We show that a two-fold elevated expression of $Cdkn1c$ alone, at levels that model loss-of-imprinting, induces a physiologically and behaviourally altered dopaminergic state. Specifically, elevated $Cdkn1c$ expression caused increased striatal $Th$ and $Dat$, elevated Th immunoreactivity
and regional specific increases in dopamine. The \textit{Cdkn1c}\textsuperscript{BACx1} animals also showed an increased \textit{cfos} reactivity to a stimulant in the ventral striatum, increased motivation to obtain a palatable reward, and increased social dominance. While we can not draw conclusive causal relationships between elevated \textit{Cdkn1c}, altered neurobiology and the observed behavioural phenotypes, this work underlines the importance of correct imprinted gene expression for brain and behaviour. Additionally, as the expression of rodent \textit{Cdkn1c} is sensitive to maternal diet (Van De Pette \textit{et al.}, 2017, Vucetic \textit{et al.}, 2010b) and post-natal care (Pena \textit{et al.}, 2014), dysregulation of this gene may underpin some aspects of the neural and behavioural phenotypic consequences associated these adverse early life environments.

In the dorsal striatum, animals over expressing \textit{Cdkn1c} were found to have an increased tissue level of dopamine, a finding emphasised by a significant increase in \textit{Dat} mRNA levels compared to WT littermates and an increase in \textit{Th} mRNA and protein expression in the ventral striatum. The increase in dopamine levels and the reuptake transporter mRNA, in absence of an increase in the metabolite DOPAC, occurs without a significant change in \textit{Th} mRNA or protein. HPLC analysis on whole postmortum tissue is not reflective of neurotransmitter dynamics \textit{in vivo} and further studies using \textit{in vivo} microdialysis may be more informative in future (Usiello \textit{et al.}, 2000). A possible underlying source for the increase in \textit{Th} in the ventral striatum is an increase in the input cell number from the VTA. This convergent evidence highlights the altered basal dopaminergic state of animals overexpressing \textit{Cdkn1c}. Given that \textit{Cdkn1c} is a cell cycle regulator a change, in addition to the observation of an increase in \textit{Th}-positive cells in the VTA, an increase in total cell number may underlie these changes. However, this did not appear be the case as total neuronal cell number was not different between \textit{Cdkn1c}\textsuperscript{BACx1} animals and their WT littermates in either the striatum or surrounding cortex. Moreover, there was no increase in \textit{Th}-positive cells in the VTA-adjacent nucleus, the SNc. The dissociation between increased \textit{Th}-positive cells of the midbrain in the absence of a global increase in neuronal cells number is not unexpected, as the function of \textit{Cdkn1c} in promotion of dopaminergic neuron proliferation is known to be independent of the CDKi domain (Joseph \textit{et al.}, 2003).
We show that a consequence of a physiologically relevant increase in *Cdkn1c* is a hypersensitivity to amphetamine, a drug of abuse in humans. This injection of amphetamine did not alter *cfos* expression in the WT littermates. This concentration of amphetamine lower than was used previously to increase locomotor activity (Mcnamara *et al.*, 2016) and plausibly was too modest for an observable effect in WT animals using this analysis technique. The location of this neural hypersensitivity is significant as it occurred specifically in the ventral striatum, containing the nucleus accumbens. A number of immediate early genes, including *cfos*, are rapidly induced in the nucleus accumbens following exposure to an addictive substance (Hope *et al.*, 1994), and this region, part of the brain’s “reward circuitry” (Russo & Nestler, 2013, Smith *et al.*, 2011, Wise, 2013) is known to undergo significant changes that underlie the behavioural transition to addiction (Nestler, 2001). The altered sensitivity of cells in this region to amphetamine emphasises the altered dopaminergic state as a consequence of elevated *Cdkn1c*.

Alterations in neural responsivity to injection of a rewarding substance were mirrored in differences in behavioural responding to a palatable food reward. *Cdkn1c* BACx1 animals had an increased motivational drive to obtain a sucrose solution. In addition, the average inter nose poke interval and time to complete a trial was shorter in *Cdkn1c* BACx1 animals. These latter measures relate to how rapidly an animal obtains successive rewards. These differences suggest that *Cdkn1c* BACx1 animals spent more time engaging in activities relating to obtaining the sucrose reward, compared to WT. We have previously demonstrated that *Cdkn1c* BACx1 animals were hypoactive in an arena in comparison to their wt littermates (Mcnamara *et al.*, 2016). However, activity levels do not appear to be a confounding factor in behaviour in this task as there was no effect of genotype on beam breaks, a measure of locomotor activity, during task performance. Bonferroni post-hoc tests demonstrated that the decrease in BP when working to obtain the calorie-free saccharin in comparison to 8% sucrose was statistically significant only in *Cdkn1c* BACx1 animals and not their WT littermates. This indicates that *Cdkn1c* BACx1 performance was more influenced by the calorific reward of sucrose and less by the palatability. However, BP in *Cdkn1c* BACx1 animals remained elevated with respect to WT littermates for saccharin,
indicating a heightened motivational drive for both caloric and non-caloric
rewards. This finding is initially at odds with our previously published work
indicating a blunted hedonic response to sucrose in animals with elevated
Cdkn1c expression (Mcnamara et al., 2016). Intriguingly, dopamine release in
dorsal and ventral striatum has recently been shown to be functionally distinct
with respect to hedonic and nutritional value sensing (Tellez et al., 2016). The
regional specificity of the changes in the dopaminergic system may reflect a
differential input magnitude to the dorsal and ventral striatum as a consequence
of elevated Cdkn1c, and may underlie the observed behavioural phenotype.

In addition, we also explored social dominance behaviour in the Cdkn1cBACx1
animals. Previous work has shown that social dominance is governed in part by
dopamine in rodents (Jupp et al., 2016) and that more dominant animals also
show increased motivation for reward (Davis et al., 2009). However, the
relationship between dopamine and social dominance is not clear-cut, and other
studies have implicated additional brain systems (Wang et al., 2011).

Nevertheless, we particularly wanted to explore this behaviour here in light of
our previous work with another imprinted gene, Grb10, where we demonstrated
that mice carrying a paternal knockout (Grb10patKO) showed increased social
dominance (Garfield et al., 2011). Mirroring the work with Grb10patKO mice,
Cdkn1cBACx1 mice were also more likely to win a tube-test encounter with
unfamiliar animals than their WT littermates. This increased success was absent
in our additional control line, Cdkn1cBACLacZ, again indicating that this behaviour
was a consequence of elevated Cdkn1c expression alone. From this dataset, we
can not exclude the possibly that the hypoactivity we have previously reported in
these animals influences performance in this task, rather than altered dominance
per se. Additionally Cdkn1c expression peaks at E13.5 (Westbury et al., 2001),
therefore, it is possible that these phenotypes are due to a general dysregulation
early in development as a consequence of elevated expression from the BAC
transgene, not limited to the dopaminergic system. It is possible to limit these
effects to a limited subset of tissues where the BAC is expressed, including the
developing nervous system (Andrews et al., 2007). Nonetheless, this is the first
explicit demonstration of a convergent role for oppositely imprinted genes on a
behavioural function in a directly comparable task, paralleling other functional
studies indicating a convergent role for imprinted genes in placental function, energy homeostasis and thermogenesis (Cleaton et al., 2014).

Current data suggests that generally imprinted genes are relatively insensitive to environmental manipulations such as in utero nutritional programming and instances of dosage regulation by relaxation of imprinting are rare and likely to be highly regulated (Radford et al., 2012). However, one such exception is Cdkn1c where expression in brain is sensitive to adverse early life events. Specifically, both low maternal dietary protein (Vucetic et al., 2010b), and the degree of post-natal maternal care (Pena et al., 2014) lead to increased Cdkn1c expression in the brain. For the case of both low maternal dietary protein this appears to be due to de-repression of the normally silent paternally inherited allele of Cdkn1c (Van De Pette et al., 2017). Both of these early life manipulations are associated with changes in the dopamine system and the response to reward (Pena et al., 2014, Vucetic et al., 2010b). Indeed the contribution of maternal diet (Ong & Muhlhausler, 2011, Vucetic et al., 2010a), and maternal care (Hall et al., 1999), to the development and function of the offspring dopamine system is well established. Whilst it is likely that the expression of many genes is affected by these adverse early life events, our findings, where Cdkn1c expression alone is elevated through genetic modification, would suggest that changes in expression of this imprinted gene could play a key role in mediating the associated brain and behavioural changes. However, the extent to which increased Cdkn1c expression mediates phenotypic changes brought about by adverse early life events, remains to be clarified.

In conclusion, we have shown that elevated expression of Cdkn1c, equivalent to loss-of-imprinting, results in an altered dopaminergic neural state and increased motivation for reward and social dominance, the latter finding providing the first example of a convergent role for imprinted genes on a behavioural function. The behavioural consequences of an altered dopamine system are further supported by our previous findings using this model where we demonstrated altered locomotor activity and sensory-motor gating deficits (Mcnamara et al., 2016). This study reemphasises the function of imprinted genes on complex adult behaviour and, as Cdkn1c expression is sensitive to the early life environment
(Pena et al., 2014, Van De Pette et al., 2017, Vucetic et al., 2010b), demonstrates a potential role of the imprinted gene Cdkn1c in mediating the changes dopaminergic function seen following such adverse early life events. Finally, this work may have also have implications for the imprinting disorders Beckwith-Wiedemann, Silver-Russell and iMAGE syndromes that are associated with altered CDKN1C dosage (Eggermann et al., 2014).

DECLARATIONS

Ethics statement
All procedures were conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986, under the remit of Home Office license number 30/9291. These procedures were also approved by the appropriate ethics committee at Cardiff University.

Competing interests
All authors declare no financial and non-financial competing interests

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Authors' contributions
GMN, RMJ and ARI designed the research. GMN, BAD and MB performed the experiments, with input from DMD and TH. JX and JWD performed HPLC analysis. GMN, RMJ and ARI wrote the paper.

REFERENCES


Figure 1: Elevated expression of Cdkn1c results in an altered dopaminergic state in the striatum in Cdkn1c<sup>BACx1</sup> animals. (A) Th immuno-reactivity in the striatum (B) Adult Th expression normalised to WT (C) Dat expression normalised to WT. Whole tissue dopamine concentration in DS (D) and VS (E). Average NeuN<sup>+</sup> cell count in striatum and cortex for Cdkn1c<sup>BACx1</sup>. Dorsal striatum (DS) ventral striatum (VS) Data shown ±SEM. *p<0.05, **p<0.01
Figure 2: Cdkn1c^{BACx1} animals display increased neural reactivity to an i.p. injection of amphetamine associated with increased mesolimbic input cell number. cfos expression in ventral striatum following a saline of amphetamine was significantly increased only in Cdkn1c^{BACx1} animals. Data shown is mean fold change ± SEM. *p<0.05 main effect of DRUG. Cdkn1c^{BACx1} had significantly more Th-positive cells than WT in VTA, but not SNc. Data shown is mean cell number per section ± SEM. *p<0.05.
Figure 3: *Cdkn1c<sup>BACx1</sup>* animals show increased motivation to obtain a sucrose reward compared to WT. (A) Average number of trials completed during CRF trials. When minimal work (i.e. a single nose poke) was required to obtain a reward there was no difference in number of trials completed between *Cdkn1c<sup>BACx1</sup>* animals and their WT littermates. (B) When the number of nose-pokes required to obtain a reward increased within a session, *Cdkn1c<sup>BACx1</sup>* made more nose-pokes that WT before stopping (BP). (C) Duration of time between successive nose-pokes was shorter in *Cdkn1c<sup>BACx1</sup>* animals. Average inter nose-pokes interval. (D) Consequently, average time to complete a trial was shorter in *Cdkn1c<sup>BACx1</sup>* animals. Data shown ±SEM. **p<0.01, ***p<0.001
Figure 4: *Cdkn1c*<sup>BACx1</sup> maintained elevated BP when presented with a less palatable sucrose concentration or a calorie-free sweetener. *Cdkn1c*<sup>BACx1</sup> animals maintained a higher average BP compared to WT when sucrose concentration was reduced from 8% to 2%. *Cdkn1c*<sup>BACx1</sup> animals maintained a higher BP in an FR2 schedule than WT animals when working for the calorie free sweetener, saccharin. Data shown ±SEM **p<0.01
Figure 5: *Cdkn1c<sup>BACx1</sup> males are more dominant in a tube test.* (A) *Cdkn1c<sup>BACx1</sup> animals won significantly more encounters against unfamiliar animals in the tubes test than wt. There was no difference in proportion of encounters won between *Cdkn1c<sup>BAClacZ</sup>* and WT animals.
Dopaminergic and behavioral changes in a loss-of-imprinting model of $Cdkn1c$

- SUPPLEMENTARY MATERIALS

Gráinne I. McNamara$^1$, Brittany A. Davis$^2$, Molly Browne$^2$, Trevor Humby$^{1,3}$, Jeffrey W. Dalley$^{4,5}$, Jing Xia$^4$, Rosalind M. John$^2$ & Anthony R. Isles$^1$*

$^1$Behavioural Genetics Group, MRC Centre for Neuropsychiatric Genetics and Genomics, Neuroscience and Mental Health Research Institute, Cardiff University, Cardiff, United Kingdom CF24 4HQ

$^2$School of Biosciences, Cardiff University, Cardiff, United Kingdom, CF10 3AX

$^3$School of Psychology, Cardiff University, Cardiff, United Kingdom CF10 3AT

$^4$Departement of Psychology, University of Cambridge, Cambridge, United Kingdom, CB2 3RQ

$^5$Department of Psychiatry, University of Cambridge, Cambridge, United Kingdom, CB2 0SZ

*Corresponding author: Email: IslesAR1@cardiff.ac.uk; Tel. +44(0)2920 688467
RESULTS

Basal dopaminergic state was not altered in Cdkn1c<sup>BAC</sup>LacZ control mice

In adult Cdkn1c<sup>BAC</sup>LacZ control mice mRNA levels in the striatum of the gene Th were unaltered relative to WT littermate (Figure S1A; main effect of GENOTYPE: F<sub>1,5</sub>=2.2, p=0.2). Similarly, expression of Dopamine transporter (Dat) mRNA was also equivalent (Figure S1B; t(8)=-0.1, p=0.923) in the dorsal striatum of Cdkn1c<sup>BAC</sup>LacZ animals compared with WT littermates. This lack of a difference in mRNA levels of key dopaminergic genes was supported by equivalence between Cdkn1c<sup>BAC</sup>LacZ animals and WT littermates in whole tissue dopamine concentration in the dorsal striatum (Figure S1C; main effect of genotype F<sub>1,14</sub>=0.481, p=0.499), and Th protein immunoreactivity in the dorsal and ventral striatum of (Figure S1D; main effect of GENOTYPE F<sub>1,6</sub>=0.617, p=0.662). Finally, there was no difference between Cdkn1c<sup>BAC</sup>LacZ animals and WT littermates in relative cell counts in the striatum (F<sub>1,7</sub>=0.025, p=0.879) or surrounding cortex (F<sub>1,7</sub>=0.732, p=0.425), as determined by number of NeuN positive cells (Figure S1E).

Motivation in a PR task was not altered in Cdkn1c<sup>BAC</sup>LacZ control mice

During CRF trials, where a single nose-poke elicited an 8% sucrose reward, there was no difference in number of trials completed between WT and Cdkn1c<sup>BAC</sup>LacZ animals (Figure S2A; main effect of GENOTYPE: F<sub>1,23</sub>=0.083, p=0.776). In a progressive ratio schedule, during which the number of nose pokes required to receive a reward ascends within a session Cdkn1c<sup>BAC</sup>LacZ animals also had an equivalent breakpoint to their WT littermates (Figure S2B; F<sub>1,23</sub>=1.012, p=0.33). The lack of difference in the main measure of motivation on this task was also reflected in additional measures, such as the inter nose-poke interval (Figure S2C; F<sub>1,23</sub>=0.236, p=0.61), and time to complete trials (Figure S2D; F<sub>1,23</sub>=0.036, p=0.85), in which Cdkn1c<sup>BAC</sup>LacZ animals performed at an equivalent level to their WT littermates.
Figure S1: *Cdkn1c*<sup>BACx1</sup> animals had elevated neural *Cdkn1c* expression at e18.5 as determined by qPCR. Data shown ±SEM **p<0.01
Figure S2: There was no effect of genotype on dopaminergic state in the striatum of Cdkn1cBACLacZ animals and WT littermates. (A) Adult Th expression normalised to WT (B) Dat expression normalised to WT. (C) Whole tissue dopamine concentration. (D) Th immuno-reactivity in the striatum (E). Average NeuN$^{+ve}$ cell count. Dorsal striatum (DS) ventral striatum (VS) Data shown ± SEM.
Figure S3: There was no effect of genotype on motivation to obtain a sucrose reward compared between $Cdkn1c^{BACLacZ}$ animals and WT littermates. (A) Average number of trials completed during CRF trials (B) BP when the number of nose-pokes required to obtain a reward increased within a session. (C) Duration of time between successive nose-pokes (D) Average time to complete a trial. Data shown ±SEM.