REVIEW

Imprinted genes and hypothalamic function

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Abstract

Genomic imprinting is an important and enigmatic form of gene regulation in mammals in which one copy of a gene is silenced in a manner determined by its parental history. Imprinted genes range from those with constitutive monoallelic silencing to those, typically more remote from imprinting control regions, that display developmentally regulated, tissue-specific or partial monoallelic expression. This diversity may make these genes, and the processes they control, more or less sensitive to factors that modify or disrupt epigenetic marks. Imprinted genes have important functions in development and physiology, including major endocrine/neuroendocrine axes. Owing to is central role in coordinating growth, metabolism and reproduction, as well as evidence from genetic and knockout studies, the hypothalamus may be a focus for imprinted gene action. Are there unifying principles that explain why a gene should be imprinted? Conflict between parental genomes over limiting maternal resources, but also co-adaptation between mothers and offspring, have been invoked to explain the evolution of imprinting. Recent reports suggest there may be many more genes imprinted in the hypothalamus than hitherto expected, and it will be important for these new candidates to be validated and to determine whether they conform to current notions of how imprinting is regulated. In fully evaluating the role of imprinted genes in the hypothalamus, much work needs to be done to identify the specific neuronal populations in which particular genes are expressed, establish whether there are pathways in common and whether imprinted genes are involved in long-term programming of hypothalamic functions.

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Introduction

Imprinted genes in mammals pose an exception to Mendelian inheritance, because one of their two copies (alleles) has been silenced in a manner predetermined by the sex of the parent transmitting the allele. There are imprinted genes for which the allele from the mother is silent and imprinted genes for which the father's allele is silent. Imprinting is a classic epigenetic phenomenon: the distinct activity states of the two alleles represent a memory of an earlier decision and can coexist throughout the lifetime of the individual, but are reversible, as imprinting is reset during passage through the germline (Ferguson-Smith & Surani 2001, Arnaud 2010). Until recently, ~100 imprinted genes had been identified in mouse and somewhat fewer in human (www.geneimprint.com), with imprinting of many conserved between the two species (Morison *et al.* 2005). A recent report, however, suggests that the figure may be substantially higher (Gregg *et al.* 2010*a*). Imprinted genes are recognised as playing important roles in foetal and placental development, growth of the foetus, peri- and postnatal physiology and in certain behaviours (Charalambous *et al.* 2007). Whether all these processes have been primarily targeted for control by imprinting or are epiphenomena the outcome is the same: having only one functional copy of a gene means that there is no backup to safeguard against mutations. Accordingly, inactivating mutations in imprinted genes or other defects that eliminate the active copy are responsible for a number of genetic syndromes (Butler 2009). Disease can also arise from 'loss of imprinting', when the normally repressed copy of an imprinted gene becomes reactivated leading to overexpression. Given these potential hazards, there must be an evolutionary benefit to imprinting, especially since many imprinted genes are shared between human and mouse, and imprinting of the insulin-like growth factor 2 (*Igf2*) and *H19* genes can be traced back to marsupials (Smits *et al.* 2008).

Silencing one allele of an imprinted gene is achieved by mechanisms that involve DNA methylation, repressive histone modifications and the actions of

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non-coding RNAs, and a great deal of progress has been made in understanding the processes that lead to the establishment and maintenance of imprinting (Weaver *et al.* 2009). Fundamental to imprinting is the establishment of differences in DNA methylation between male and female gametes at imprinting control regions (ICRs), and these primary imprint marks persist throughout the lifetime of an individual and are only erased and reset in the germline. Most imprinted genes occur in clusters that span several hundred kilobases and monoallelic expression of all genes in a cluster is determined *in cis* by a single ICR. Whilst the primary epigenetic marks at ICRs are maintained during the lifetime, not all imprinted genes are expressed monoallelically throughout life. The human *IGF2* gene is expressed predominantly from the paternal allele in the foetus and placenta, but from both alleles equally in the liver after birth (Vu & Hoffman 1994). *GNAS*, which encodes the stimulatory G protein α subunit Gsα, exhibits paternal allele silencing in a limited set of tissues, including the pituitary, thyroid and hypothalamus (Hayward *et al.* 2001, Mantovani *et al.* 2002, Liu *et al.* 2003, Germain-Lee *et al.* 2005, Chen *et al.* 2010). This may suggest that imprinted expression for a widely expressed gene with pleiotropic effects (as a common mediator of G protein-coupled receptor signalling) has been selected only for a subset of the functions it serves. Stage- or tissue-specific imprinting might be the consequence of the fact that most imprinted genes in clusters are not directly repressed by the monoallelic DNA methylation present at ICRs, but rely on mechanisms such as silencing by non-coding RNAs that originate from ICRs (Weaver *et al.* 2009). Such indirect mechanisms may also allow a degree of plasticity in imprinting, but the possibility that there is interindividual variation in imprinting deserves further investigation (Turan *et al.* 2010). It is important to stress that whether or not monoallelic expression is maintained, the primary imprint marks at ICRs remain in place.

The hypothalamus as a hotspot of imprinted gene expression

The findings that mouse knockouts for several imprinted genes have disturbed metabolic control highlights the hypothalamus as a potential major site for the action of imprinted genes (Charalambous *et al.* 2007, Frontera *et al.* 2008). The hypothalamus is a key regulator of many endocrine functions and is involved in the regulation of energy balance through its influence on food intake, metabolic rate and body temperature by the action of a dozen small nuclei. It has well-defined circuits between the nuclei that allow integration and fine-tuning of the systems responsible for homeostasis and adaptation to environmental change. Developmental studies using aggregation chimaeras in mice, in which parthenogenetic (PG) or androgenetic cells (AG) were combined with normally fertilised embryos, provided evidence for a role of imprinted genes in brain development, including the hypothalamus. PG embryos are produced by activation of unfertilised oocytes and contain no paternal genetic contribution; AG embryos by pronuclear transplantation in one-cell embryos and contain two paternal genomes. AG and PG cells are generally at a disadvantage during embryonic development, but in the brain AG cells become restricted to the hypothalamus, whereas PG cells are excluded and are found predominantly in the cortex (Allen *et al.* 1995, Keverne *et al.* 1996). This work graphically illustrated that paternally and maternally expressed imprinted genes might have distinct impacts on brain development and function, suggesting, for example, that paternally expressed gene function may predominate in regions associated with primary motivated behaviours (Keverne *et al.* 1996). A number of imprinted genes are now known to be expressed in hypothalamic nuclei and could be involved in establishing hypothalamic neuroarchitecture and neural circuits. In an extensive survey of imprinted gene expression in mouse brain, it was found that certain regions, in particular the hypothalamus, express a higher proportion of the known imprinted genes (Gregg *et al.* 2010*a*). Neural systems associated with feeding and metabolism, and motivational behaviours emerged as 'hotspots' for imprinted genes.

Imprinted gene disorders with a neuroendocrine involvement

The involvement of imprinted genes in neuroendocrine and endocrine function was first suggested by the discovery of the imprinted basis of disorders such as the Prader–Willi syndrome (PWS) and Albright hereditary osteodystrophy (AHO). PWS is a complex neuroendocrine and behavioural disorder that presents in infants with hypotonia and feeding difficulties, followed by hyperphagia and undiscerning eating, development of morbid obesity, hypogonadism and cognitive delays (Cassidy & Driscoll 2009). Patients present with low levels of testosterone, gonadotropins, GH and IGF1 (Miller *et al.* 2008); they are also deficient in the anorectic peptide YY and have elevated levels of gut peptides obestatin and ghrelin, but normal thyroid and adrenal function, suggesting primary hypothalamic rather than endocrine dysfunction. There are reports of hypothalamic abnormalities in patients, including reduced numbers of oxytocin-producing neurons (Swaab 1997). The PWS region on chromosome 15q11–q13 is one of the most complex imprinted

domains and comprises numerous paternally expressed imprinted genes, suggesting that full-blown PWS is best considered a contiguous gene syndrome. The region also encodes multiple small nucleolar RNAs (snoRNA), particularly of the C/D box class, which are involved in RNA processing, and recent genetic analysis in patients (Sahoo *et al.* 2008, de Smith *et al.* 2009) and knockout studies in mice (Ding *et al.* 2008, 2010) implicate disruption of specific snoRNAs as a major factor in PWS.

AHO, an autosomal dominant disorder caused by inactivating mutations in *GNAS*, represents the classical endocrine imprinted disorder. Characteristics include short stature, brachymetacarpia, subcutaneous ossification and developmental delay, but a remarkable feature is that presentation depends on parental origin (Davies & Hughes 1993, Wilson *et al.* 1994). When transmitted from mothers, AHO is accompanied by the hormone resistance syndrome pseudohypoparathyroidism type 1a (PHP1a), characterised by development of end-organ resistance to a subset of hormones – parathyroid hormone, GHRH, TSH and gonadotropins – which depend on Gsα-coupled receptors (Weinstein 1998). AHO may be explained by generalised haploinsufficiency of *GNAS*; PHP1a represents the additional consequences of tissue-specific imprinting of *GNAS* – the relative silencing of the paternal allele – such that in some tissues Gsα function is effectively absent or severely reduced when the maternal allele carries a mutation. The imprinted aetiology of PHP1a is underlined by the related disorder PHP1b, in which much of the spectrum of hormone resistance typical of PHP1a occurs in the absence of the physical features of AHO (Kelsey 2010). PHP1b is caused by epigenetic defects of *GNAS* imprinting rather than inactivating mutations (Liu *et al.* 2000, Bastepe *et al.* 2001). PHP1a patients also develop obesity (Long *et al.* 2007) but, since *GNAS* expression is not imprinted in adult adipose tissue (Mantovani *et al.* 2004, Chen *et al.* 2010), Gsα function in adipocytes is not a major contributor to this metabolic phenotype, despite the key role of Gsα in regulating lipid mobilisation. Instead, evidence from tissue-specific ablation of *Gnas* in mouse implicates imprinted expression in the hypothalamus as the cause (Chen *et al.* 2009).

Studies of imprinted gene function in the hypothalamus

For a number of imprinted genes, knockouts have revealed defects in hypothalamic development and function (Table 1). Amongst imprinted genes in the PWS domain in 15q11–q13, evidence from mouse studies indicates that several are expressed in the hypothalamus and their deficiency may contribute to some aspects of the complex PWS phenotype. *Magel2* encodes a putative transcriptional regulator expressed predominately in the brain and especially during late developmental stages in the hypothalamus (Kozlov *et al.* 2007). *Magel2*-deficient mice exhibit neonatal growth retardation, altered metabolism and increased adiposity, despite a reduction in food intake (Bischof *et al.* 2007, Kozlov *et al.* 2007). They have reduced numbers of orexin neurons and orexin levels in the lateral hypothalamus, suggesting a role for *Magel2* in neuronal development. There is accumulation of oxytocin intermediates in hypothalamus of neonates and injection of oxytocin rescues the feeding impairments and lethality (Schaller *et al.* 2010). *Ndn* encodes necdin, a potent post-mitotic growth suppressor and anti-apoptotic factor predominately expressed in differentiated neurons (Muscatelli *et al.* 2000). Necdin is highly expressed during development of the nervous system and interacts with neurotrophic receptors. Several knockout models of *Ndn* have been reported with different phenotypic outcomes ranging from early postnatal lethality owing to respiratory distress to normal viability (Gérard *et al.* 1999, Muscatelli *et al.* 2000, Kuwako *et al.* 2005). *Ndn* knockout mice variously demonstrate hypothalamic changes, including reduced numbers of oxytocin- and LHRH neurons (Muscatelli *et al.* 2000), more generalised defects in neuronal differentiation (Kuwako *et al.* 2005, Kuwajima *et al.* 2006) and abnormally high levels of serotonin in the medulla (Zanella *et al.* 2008). Necdin is also implicated in axonal outgrowth, routing and fasciculation, thereby possibly participating in establishment of neuroendocrine circuits (Lee *et al.* 2005).

Besides genes associated with PWS, several other imprinted genes are implicated in regulating hypothalamic functions. The paternally expressed gene for neuronatin (*Nnat*) is expressed abundantly in various hypothalamic nuclei. Expression is downregulated after fasting and in genetic models of obesity (*ob/ob* mice), and is responsive to leptin administration, suggesting an involvement in regulating energy homeostasis. No knockout of *Nnat* has been reported, but in humans single nucleotide polymorphisms in *NNAT* are associated with severe childhood and adult obesity (Vrang *et al.* 2010). Of additional imprinted genes with hypothalamic functions, *Gnas* and *Peg3* are dealt with in detail below. Together, these observations suggest that a number of imprinted genes expressed in the hypothalamus from early developmental stages participate in establishing neuronal circuits responsible for fine-tuning and coordinating the system to anticipate appropriate responses to the environment.

Table 1 Imprinted gene knockouts in the mouse demonstrating hypothalamic effects

Imprinted gene	Expressed allele	Function of the gene product	Knockout phenotype	References
Magel2	Paternal	Putative transcription factor	Neonatal growth retardation, excessive post-weaning weight gain, increased adiposity and altered metabolism	Bischof *et al.* (2007)
			Reduced amplitude of activity and increased daytime activity, reduced food intake, orexin levels and number of orexin-positive neurons	Kozlov *et al.* (2007)
Ndn	Paternal	Post-mitotic growth suppressor	Early postnatal lethality	Gérard *et al.* (1999)
			Neonatal lethality, reduction in oxytocin- and LHRH-producing neurons in hypothalamus	Muscatelli *et al.* (2000)
			Impaired neuronal development and differentiation	Kuwako *et al.* (2005) and Kuwajima *et al.* (2006)
Gnas	Maternal allele-specific expression limited to specific tissues (PVN)	Stimulatory G protein α subunit	Constitutive knockouts (maternal transmission)	
			Higher birth weight, TSH and PTH resistance	Germain-Lee *et al.* (2005)
			Reduced metabolic rate, energy expenditure and locomotor activity, severe obesity, insulin resistance and impaired glucose tolerance	Chen *et al.* (2005)
			Brain-specific knockout (maternal transmission)	
			Obesity, reduced metabolic rate, impaired melanocortin response and insulin resistance	Chen *et al.* (2009)
Gnasxl	Paternal	Extra-large isoform of stimulatory G protein α subunit	Decreased adiposity and body weight, elevated metabolic rate, improved glucose tolerance and insulin sensitivity	Xie *et al.* (2006)
Peg3	Paternal	Zinc finger transcription factor	Foetal growth retardation, maternal behaviour impairment, altered neuropeptide balance and reduction in oxytocin-positive neurons	Li *et al.* (1999)
			Growth retardation, lower core body temperature, reduced metabolic rate, increased adiposity, elevated leptin, leptin resistance, altered hypothalamic neuropeptide expression and later onset of puberty	Curley *et al.* (2005)

Reasons for imprinting in the hypothalamus

Why should the hypothalamus be a hotspot for imprinted genes? A number of explanations for the evolution of imprinting in mammals have been advanced (as far as we know, amongst vertebrates genomic imprinting is restricted to placental mammals), the most pertinent to hypothalamic function appear to be 'conflict' and 'co-adaptation'. The 'conflict hypothesis' posits that the inequality in parental investment in placental mammals and opportunity for paternal genes to manipulate the amount of resources offspring obtain from mothers, together with the likelihood that these paternal genes are not related to paternal genes in future offspring from the same female, gives rise to different selective pressures on paternal and maternal genes in offspring. Paternal genes can be considered resource demanding, whilst maternal genes in offspring have an interest in conserving maternal resources for the future reproductive health of the mother (Moore & Haig 1991). Most imprinted genes that influence foetal and early postnatal growth and development satisfy the predictions of conflict theory, but the theory has had less success in predicting the actions of imprinted genes in adults, and there seems to be no neat division between the effects of maternally and paternally expressed genes in processes such as metabolism (Haig 2004, Frontera *et al.* 2008).

The *Gnas* locus exemplifies the possibility of conflict because of the opposing effects of maternally and paternally expressed gene products. The canonical *Gnas* transcript encoding Gsα is expressed with a highly cell-type pattern of silencing of the paternal allele. But there is an isoform of Gsα, called XLαs, that is produced exclusively by the paternal allele. The XLαs-encoding transcript *Gnasxl* is determined by an alternative, upstream promoter, such that XLαs contains essentially all the functional domains of Gsα, which are encoded by shared exons, but XLαs has a specific, large amino-terminal domain (Kehlenbach *et al.* 1994).

XLαs stimulates adenylyl cyclase in response to receptor activation in a similar manner to Gsα (Bastepe *et al.* 2002), although it may interact more avidly with the plasma membrane and be sensitive to lower ligand concentrations (Kaya *et al.* 2009). In some circumstances *in vivo* XLαs can replace Gsα (Liu *et al.* 2011). In contrast to similar biochemical properties, at a physiological level Gsα and XLαs appear to act in opposing pathways. This was intimated by the first gene knockout of *Gnas*, which targeted exon 2 common to both Gsα- and XLαs-producing transcripts. The exon 2 disruption causes severe phenotypes in heterozygotes but, remarkably, these differ according to whether the mutation is transmitted maternally or paternally (Yu *et al.* 1998). Mice lacking maternal Gsα expression are hypometabolic and become obese, with decreased activity and energy expenditure, on account of reduced activity of the sympathetic nervous system; mice with the paternal mutation are lean and hypermetabolic owing to increased sympathetic activity (Yu *et al.* 2000). Further analysis of mice with disruption specifically of Gsα (deletion of the Gsα-specific exon 1) or of XLαs attributes the maternal hypometabolic phenotype to lack of Gsα and the paternal hypermetabolic phenotype to lack of XLαs (Chen *et al.* 2005, Xie *et al.* 2006). Recent work on a conditional knockout of Gsα establishes that the metabolic phenotypes are determined in the central nervous system (Chen *et al.* 2009): ablating Gsα in neurons reproduces the hypometabolic phenotype, but only in heterozygotes lacking Gsα from the maternal allele. The central melanocortin system is a major regulator of metabolic rate and the melanocortin-4 receptor is Gsα coupled. Brain-specific maternal Gsα-deficient mice have a blunted metabolic response to melanocortins even before the onset of obesity. *In situ* hybridisation revealed imprinted expression of Gsα in the hypothalamus, specifically in the paraventricular nucleus (PVN), whereas in other brain regions expression from both alleles is similar. Specific deletion of Gsα in the PVN will be required to show definitively that imprinted expression in this region is involved in regulating metabolic rate. In contrast to Gsα, XLαs has a far more restricted domain of expression, including the hypothalamus, and is strictly imprinted. The extent to which the lean, hypermetabolic phenotype of XLαs-deficient mice has a basis in hypothalamic expression of XLαs is unclear, as the existing knockout is a constitutive deletion. However, XLαs is expressed predominantly within orexigenic neurons (E Ivanova, G Kelsey and M Frontera, unpublished observations), giving rise to the possibility that XLαs may function as the major Gsα isoform in hypothalamic regions suppressing metabolic rate and is involved preferentially in transducing signals from receptors for energy preserving pathways. It will be important to investigate how the respective phenotypes are modified when Gsα and XLαs deficiencies are combined to establish whether these oppositely imprinted gene products indeed have antagonistic functions compatible with the conflict theory.

An alternative hypothesis considers that imprinted expression can arise because of 'co-adaptation' of maternal and offspring traits in the interests of achieving optimal birth weight and fitness of offspring, to ensure not only that offspring extract optimal resources from females but that females are capable of optimal provisioning their young (Wolf & Hager 2006). The paternally expressed gene *Peg3*, which encodes a Krüppel-type zinc finger transcription factor involved in apoptotic pathways (Relaix *et al.* 1998, Kohda *et al.* 2001), is an exemplar of this concept. *Peg3* is expressed from early embryogenesis (Kuroiwa *et al.* 1996); in the brain, it is prominent in the arcuate, ventromedial, dorsomedial, paraventricular and some other hypothalamic nuclei. Deficiency of *Peg3* results in foetal growth retardation (Li *et al.* 1999). In adult *Peg3* knockout mice the balance of feeding peptides is changed in the hypothalamus and sympathetic activity is decreased, as reflected in lower core body temperature and metabolic rate and increased adipose tissue mass (Curley *et al.* 2005). In addition, *Peg3* knockout mice have reduced numbers of oxytocin-positive neurons, and females exhibit impaired milk ejection and deficits in maternal care (Li *et al.* 1999). It is possible that *Peg3* is essential for development of neuronal circuitry and establishment of normal hypothalamic function, not only for the metabolic needs of the individual, but also to prime the mother for successful pregnancy and to optimise postpartum mother:offspring interactions. In the view of co-adaptation, the expression domains of *Peg3* in the embryo and the mother work in concert: *Peg3* helps shape development of the hypothalamus *in utero* at a time when, and in part because, it is also operating in the placenta to promote nutrient acquisition from the mother. The outcome is that offspring that have grown well and extracted optimal resources will be destined to be good mothers as well (Keverne 2009). With the focus on offspring:mother interactions, sexually dimorphic expression of *Peg3* in the adult hypothalamus might be expected, but no information is available. At this point, it is difficult to conclude whether co-adaptation or conflict is the major driver for imprinted genes in the hypothalamus, and it may be that different selective pressures have driven the evolution of imprinting at different loci (Wolf & Hager 2006).

How many genes are imprinted in the hypothalamus?

In the past 20 years, over 100 imprinted genes have been discovered in the mouse (www.geneimprint.com) and it is possible that most imprinted genes with major

phenotypic effects (e.g. embryo growth and viability) have been accounted for (Cattanach & Beechey 2004). However, it is likely that others exist, especially genes with temporally or cell-type restricted imprinting (Schulz *et al.* 2009), but these require more effort to locate. One promising approach is transcriptome sequencing, using next generation sequencing (mRNA-Seq), of tissues from hybrid mice (Babak *et al.* 2008, Gregg *et al.* 2010*a*,*b*). The principle is that mRNA-Seq at sufficient sequencing depth quantifies allelic expression on the basis of differences in read numbers of sequences containing parental allele variants, given appropriate statistical tests. Using reciprocal crosses (i.e. if one cross is between strain A female and strain B male, the reciprocal is strain B female with strain A male), skewed allele representation attributed to a parent-of-origin effect can be distinguished from expression bias owing to genetic difference between alleles. Moreover, the resolution of sequencing allows differential imprinting of alternative transcripts of the same gene to be discriminated. In studies reported by Gregg *et al.* (2010*a*), 256 new candidate imprinted genes (and additional non-coding transcripts) were identified in the adult mouse hypothalamus (preoptic area). Interestingly, there was a pronounced bias towards paternally expressed genes and rather little overlap with the smaller number of imprinted candidates identified in another brain area, the prefrontal cortex, recalling the preferential survival of AG cells in the hypothalamus in chimaera studies (Allen *et al.* 1995, Keverne *et al.* 1996). Extensive validation studies will need to be done to conclude how many candidates correspond to new imprinted genes, and the number displaying apparently partial allelic silencing may suggest a proportion of false positives, but the approach correctly scored the majority of known imprinted genes expressed in the brain, including cases of highly isoform-specific monoallelic expression, such as the *Inpp5f* gene. In addition to 'conventional imprinting', a substantial number of autosomal genes were identified that exhibited sex-specific biases in parental allele expression (Gregg *et al.* 2010*b*). In the adult hypothalamus, many more 'sex-specific imprinted genes' were predicted in females than males, possibly reflecting the sexually dimorphic nature of the hypothalamus and its involvement in the control of maternal and mating behaviours, and the influence of known imprinted genes on maternal behaviour (e.g. *Peg3*). Concerning the functions of the new candidates, gene ontology analysis identified cell adhesion as the most highly enriched process in the adult hypothalamus (which might be skewed by several protocadherin genes showing parent-of-origin effects) and metabolic processes for candidates in embryonic brain. Enrichment in cell adhesion molecules could support a role of imprinted genes in controlling neural architecture.

Conclusions and perspectives

It is clear from theoretical concepts and experimental observations of a handful of genes that the hypothalamus is an important site of action of imprinted genes, and disruption in normal hypothalamic expression may contribute to imprinted gene syndromes. It is too soon to evaluate the significance of the discovery of several hundred new imprinted gene candidates in the hypothalamus, including those with sex-limited imprinting, and comprehensive validation studies are required as well as investigating the basis of their monoallelic expression. We also need much more refined analysis, for instance, on the identity of neuronal subtypes expressing the various imprinted genes to be able to assess the extent to which imprinted genes function in common pathways. Systematic studies, such as transcriptome sequencing of defined neuronal subpopulations from hybrid mice, would be beneficial. Up to now, no imprinted gene has been ablated specifically in the hypothalamus, and this type of approach will be important to separate functions in the hypothalamus from possible confounds from other or earlier developmental effects of genes with pleiotropic functions. The extent to which imprinted genes are involved in developmental modelling of the hypothalamus needs further study, as well as the degree to which the epigenetic control of imprinted genes is responsive to extrinsic signals in a manner that may have lasting impacts on hypothalamus function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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