Gene Dosage Effects at the Imprinted Gnas Cluster

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Abstract

Genomic imprinting results in parent-of-origin-dependent monoallelic gene expression. Early work showed that distal mouse chromosome 2 is imprinted, as maternal and paternal duplications of the region (with corresponding paternal and maternal deficiencies) give rise to different anomalous phenotypes with early postnatal lethalities. Newborns with maternal duplication (MatDp(dist2)) are long, thin and hypoactive whereas those with paternal duplication (PatDp(dist2)) are chunky, oedematous, and hyperactive. Here we focus on PatDp(dist2). Loss of expression of the maternally expressed Gnas transcript at the Gnas cluster has been thought to account for the PatDp(dist2) phenotype. But PatDp(dist2) also have two expressed doses of the paternally expressed Gnasxl transcript. Through the use of targeted mutations, we have generated PatDp(dist2) mice predicted to have 1 or 2 expressed doses of Gnasxl, and 0, 1 or 2 expressed doses of Gnas. We confirm that oedema is due to lack of expression of imprinted Gnas alone. We show that it is the combination of a double dose of Gnasxl with no dose of imprinted Gnas, that gives rise to the characteristic hyperactive, chunky, oedematous, lethal PatDp(dist2) phenotype, which is also hypoglycaemic. However PatDp(dist2) mice in which the dosage of the Gnasxl and Gnas is balanced (either 2:2 or 1:1) are neither dysmorphic nor hyperactive, have normal glucose levels, and are fully viable. But PatDp(dist2) with biallelic expression of both Gnasxl and Gnas show a marked postnatal growth retardation. Our results show that most of the PatDp(dist2) phenotype is due to overexpression of Gnasxl combined with loss of expression of Gnas, and suggest that Gnasxl and Gnas may act antagonistically in a number of tissues and to cause a wide range of phenotypic effects. It can be concluded that monoallelic expression of both Gnasxl and Gnas is a requirement for normal postnatal growth and development.

Introduction

Early work showed that certain chromosomal regions can lead to developmental abnormalities when both copies are exclusively maternally or paternally derived. Distal mouse chromosome 2 was one of the first such imprinting regions described, providing evidence that imprinting must affect expression of some genes according to parental origin [1]. Mice with two maternally derived copies of the region but no paternally derived ones, MatDp(dist2), had long thin bodies, failed to suckle, became inert and died within a few hours of birth. On the other hand, mice with two paternally derived copies of the region, but none that were maternally derived, PatDp(dist2), had an apparently opposite phenotype for they had short square bodies, (probably due to the combined effects of oedema and a chunky body shape), were notably hyperactive and died within a few days of birth. Hyperactivity may not be evident at birth but generally developed during the day of birth and became more pronounced in following days. In addition many PatDp(dist2) mice have an unusual front foot movement described as paddle feet, and a tail kink characteristically occurring about halfway down the tail [1,2]. From genetic approaches using reciprocal translocations the region on mouse chromosome 2 giving rise to these imprinting effects was estimated to be 7 Mb in size and contained the Gnas cluster [3].

Misexpression of transcripts at the imprinted Gnas cluster can account for much of the phenotype in both MatDp(dist2) and PatDp(dist2). Gnas is a complex imprinted gene cluster with three promoter regions that give rise to protein coding transcripts Nesp, Gnaaxd, and Gnas. Each of these transcripts has a unique first exon that splices on to a common set of downstream exons (Figure 1). Nesp is exclusively expressed from the maternal allele and Gnasxl from the paternal allele. Gnas is biallelically expressed in most tissues but is preferentially maternally expressed in some [4,5,6,7]. Nesp gives rise to NESP55, neuroendocrine secretory protein 55, Gnas to Gsα, the alpha subunit of the Gs signalling protein and Gnasxl to XLαs, a variant form of Gsα which can also function as the alpha subunit of the heterotrimeric Gs protein. Both XLαs and Gsα can act on adenylyl cyclase to induce cyclic AMP production [8]. Although XLαs and Gsα share biochemical properties there is evidence that they act antagonistically and have opposite effects on metabolic regulation [9,10,11]. Overall the effects of paternally expressed Gnasxl in offspring result in increased demands on the
mother (growth promoting) whereas the effects of maternally expressed Gnas result in fewer demands, in accordance with the conflict hypothesis [12].

Imprinted expression of protein coding transcripts at the Gnas cluster is controlled by an imprinting control region (ICR), a germline differentially methylated region called the Nespas DMR that covers the promoter of a noncoding antisense transcript, Nespas [13]. The Nespas DMR is part of a larger DMR that covers the Gnasxl promoter [14]. Paternal inheritance of the Nespas\textsuperscript{im\textsubscript{117}} allele (hereafter called \(\Delta\)Nespas), a deletion of the Nespas DMR, results in loss of imprinting of Nes and Gnas, and down regulation of Gnasxl [13]. The tissue specific imprinted expression of the Gnas transcript is controlled by a second differentially methylated region, the Exon 1A DMR that lies just upstream of Gnas exon 1 [15]. Paternal inheritance of the Gnas\textsuperscript{im\textsubscript{117}} allele (hereafter called \(\Delta\)Exn1A), a deletion of the Exon 1A DMR, results in loss of imprinted expression of Gnas and so the Exon 1A DMR solely regulates the imprinted expression of Gnas [6,16]. Thus the Nespas DMR must interact with the Exon 1A DMR to control the imprinted expression of Gnas although the mechanism of interaction is not yet clear.

PatDp(dist2) mice have two expressed copies of Gnas and lack expression of Nes in imprinted tissues and also lack expression of Nesp [6,13,17] whereas MatDp(dist2) mice have two expressed copies of Gnas in imprinted tissues and two expressed copies of Nesp but lack expression of Gnasxl. PatDp(dist2) mice have greatly diminished expression of Gnas in newborn brown fat consistent with imprinted expression [6] and elevated levels of Gnasxl consistent with two expressed doses [13].

From studies of targeted deletions and an ENU induced mutation it is evident that both XLas and Gs\(z\) play important roles in growth, behaviour and survival shortly after birth, but NESP55 does not [7,9,10,18,19]. Paternal inheritance of a null mutation in Gnasxl results in newborns with severely reduced suckling ability that become inert on the day of birth and most die within a few days of birth [10]. Thus loss of expression of Gnasxl can account for much of the phenotype observed in MatDp(dist2) mice. On the other hand, maternal transmission of a null mutation resulting in loss of Gnas transcript gives rise to neonates with oedema and square shaped bodies, most of which die before weaning [7,9] and maternal inheritance of a loss of function allele, Gnas\textsuperscript{Oedsml-mat}, results in gross neonatal oedema and pre-weaning lethality [18]. PatDp(dist2) are oedematous at birth [1,20] (but the oedema is of considerably less severity than in Oeds mice) [18] and oedema may account for at least part of the original description of a square shaped body [1,21]. Thus the phenotype of mutants affecting Gzs function or expression has some similarity to that seen in PatDp(dist2). But, neither the null nor the loss of function mutation results in the hyperactivity shortly after birth that is probably the most distinctive feature of PatDp(dist2). So loss of maternal expression of Gnas transcripts in imprinted tissues can account for the oedematous phenotype seen in PatDp(dist2) mice but cannot account for the hyperactivity. Thus hyperactivity may be due to a double dose of Gnasxl alone or to the combined effect of a double dose of Gnasxl and loss of Gnas in imprinted tissues.

We have used genetic approaches to show the combination of over expression of Gnasxl and loss of Gnas gives rise to an early hyperactivity lethal phenotype. Furthermore when the expressed dosage of Gnas and Gnasxl is balanced then PatDp(dist2) are neither lethal nor hyperactive. Thus the dosage of Gnas and Gnasxl needs to be balanced for long term survival and normal activity. Furthermore PatDp(dist2) with balanced dosage but two doses of both Gnasxl and Gnas have normal viability and activity but preweaning growth retardation followed by catch up growth. Thus it may be important to have a single dose of Gnasxl and Gnas for normal development or there may be other genes involved in determining the PatDp(dist2) phenotype.

Materials and Methods

Ethics Statement

All mouse studies were carried out in accordance with the guidance issued by the Medical Research Council in “Responsibility in the use of animals in bioscience research (May 2008)”, were approved by the MRC Harwell ethical review committee and carried out under the authority of the UK Home Office.
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Mice

Paternal duplication (PatDp(dist2)) mice were generated by the standard genetic method of intercrossing genetically marked translocation heterozygotes and identifying PatDp(dist2) mice with the aid of markers [22]. From these intercrosses up to 16% of live births are expected to be PatDp(dist2) [23]. We used the translocation T(2;8)26H, (T26H), which has a breakpoint on chromosome 2 at the nonagouti locus [23] proximal to the Gnas cluster.

To generate PatDp(dist2) with altered dosing of Gsx and XLz we developed translocation stocks that were heterozygous for $\Delta Ex1A$ (T26H +/- $\Delta Ex1A$) (maternal allele listed first), homozygous for $\Delta Ex1A$ (T26H $\Delta Ex1A$/ $\Delta Ex1A$), heterozygous for $\Delta Nespas$ (T26H +/- $\Delta Nespas$), and homozygous for both $\Delta Ex1A$ and $\Delta Nespas$ (T26H+$\Delta Ex1A$/$\Delta Nespas$ +). PatDp(dist2) that were either heterozygous for $\Delta Ex1A$, or homozygous for $\Delta Ex1A$, or heterozygous for $\Delta Nespas$, or homozygous for both $\Delta Ex1A$ and $\Delta Nespas$ were generated by crossing T26H +/- females with either TH26+/+ $\Delta Ex1A$, or T26H $\Delta Ex1A$/ $\Delta Ex1A$ or T26H +/- $\Delta Nespas$ or T26H+$\Delta Ex1A$/$\Delta Nespas$+ females. For each cross, reciprocal crosses were also set up to generate PatDp(dist2) with wild-type alleles at $\Delta Ex1A$ and/or $\Delta Nespas$. The genetic background of PatDp(dist2) mice was mixed with major contributions from C3H/HeH (circa 45%) and the Harwell LL stock (circa 45%). Toe or tail clips were taken from offspring at birth and these served as both biopsies for identification and for genotyping. Offspring were genotyped for parental duplication, $\Delta Ex1A$ and $\Delta Nespas$ as previously described [6,13,22].

Phenotyping

Mice were classified for oedema, chunky appearance, activity, tail kink, and paddle feet, a paddling motion of the front feet, by daily visual observation from birth. Mice were removed from the box and observed for 2–3 minutes.

RNA Analysis

Total RNA was extracted and northern analysis carried out as described previously [6, 13]. Riboprobes specific for the unique first exons of Gnas and Gnasxl and for Actb have been previously described [6,13]. Transcript levels were measured by phosphoimager analysis and quantified as described previously [6,13].

Biochemical Analysis of Plasma

Truncal blood from mice up to one week of age was collected using heparinised capillary tubes. Plasma was separated following centrifugation at 750 x g for 3 minutes and stored at -80°C. Noradrenaline was measured in duplicate using an ELISA kit (Labor Diagnostika Nord GmbH & Co.KG) according to the manufacturer’s instructions. Blood glucose was measured using an Alphatrak Veterinary Blood Glucose Monitoring Meter Kit (Abbott) according to the manufacturer’s instructions. Insulin and glucagon were measured using an ELISA kit (Mercodia) according to the manufacturer’s instructions.

Weight and Body Mass Index

Mice were weighed weekly from birth and from 4 weeks the length from the tip of the nose to the base of the tail was measured weekly. Body mass index was calculated using the following equation: weight (g)/length (cm)$^2$.

Metabolic Caging

At nine weeks the mice were individually housed in metabolic cages for 24 h, during which time they had free access to a known amount of food and water. After 24 h, the amount of food and water consumed was measured along with the volume of urine produced. After housing in metabolic cages the mice were returned to their home cage. For a fuller protocol, see EMPRess (the European Phenotyping Resource for Standardised Screens from EUROMORPHIA; http://empress.har.mrc.ac.uk).

Metabolic Rate Measurements

At 10 and 21 weeks of age the mice were weighed and given a recoverable anaesthetic prior to scanning using a General Electric Medical Systems Lunar PIXIimus H X-ray densitometer (Inside Outside Sales, Fitchburg, WI, USA), which allows the fat and lean mass and bone density to be calculated. Mice were placed in a heated box to aid recovery; once fully recovered, they were returned to their home cage.

DEXA Analysis

At 17 weeks of age the mice were weighed and given a recoverable anaesthetic prior to scanning using a General Electric Medical Systems Lunar PIXIimus H X-ray densitometer (Inside Outside Sales, Fitchburg, WI, USA), which allows the fat and lean mass and bone density to be calculated. Mice were placed in a heated box to aid recovery; once fully recovered, they were returned to their home cage.

Statistical Analyses

Fisher’s exact test was used for comparisons of the incidence of PatDps and the occurrence of phenotypic features. Student’s t test (two-tailed) was used for assessing the results of the growth and metabolism studies and expression levels on northern blots. P values <0.05 were considered significant.

Results

Incidence and Survival of PatDp(dist2)

PatDp(dist2) has two expressed doses of Gnasxl and severely reduced expression of imprinted Gnas [6,13] and will hereafter be referred to as PatDp(dist2)/0. To produce PatDp(dist2) with differing doses of Gnasxl and Gnas we utilised two mutants, $\Delta Ex1A$ and $\Delta Nespas$. $\Delta Ex1A$ is a deletion of the Exon 1A DMR, the region that regulates the imprinted expression of Gnas. On paternal inheritance Gnas is completely derepressed on the paternal allele [6]. Heterozygotes +/- $\Delta Ex1A$ (maternal allele listed first) show normal survival to birth for they were generated at a frequency of 44.5% (of 231 neonates) which is not significantly different from the expected 50% (P>0.05). The $\Delta Nespas$ mutant allele is a deletion of the promoter and first exon of Nespas, the ICR for the Gnas cluster [13]. On paternal inheritance of $\Delta Nespas$ Gnasxl is downregulated to about 24% of wild type levels (Figure S1) and Gnas is expressed to about 70% of wild type on the paternal allele. Heterozygotes +/- $\Delta Nespas$ also show normal survival to birth [13].

Four crosses were set up to generate PatDp(dist2) mice with altered dosing of Gnasxl and Gnas (Figure 2, Table 1). In Cross 1 PatDp(dist2) mice heterozygous for $\Delta Ex1A$ were produced to provide PatDp(dist2)/2:1 with two predicted expressed doses of Gnasxl and one expressed dose of Gnas. In Cross 2 PatDp(dist2) mice homozygous for $\Delta Ex1A$ were generated to produce PatDp(dist2)/2:2 with two predicted expressed doses of Gnasxl and two doses of Gnas. Cross 3 gave rise to PatDp(dist2) mice heterozygous for $\Delta Nespas$ predicted to have slightly more than
one expressed dose of Gnasxl and slightly less than one expressed dose of Gnas, called PatDp(dist2):1:1. From Cross 4 PatDp(dist2) arose that were compound heterozygotes for ΔEx1A and A Nevps predicted to have slightly more than one expressed dose of Gnasxl and slightly less than two expressed doses of Gnas, called PatDp(dist2):1:2. For each cross reciprocal crosses were also set up resulting in PatDp(dist2):2:0.

Heterozygotes +/-ΔEx1A were known to express two doses of imprinted Gnas, and PatDp(dist2):1:2 and PatDp(dist2):1:1 to express one dose of imprinted Gnas [6]. From northern analysis, similar levels of Gnas were found in newborn brown fat from +/-ΔEx1A and PatDp(dist2):2:2 homozygous for ΔEx1A indicating that PatDp(dist2):2:2 express two full doses of Gnas (Figure 3).

There were no significant differences in the incidence or phenotypes of PatDp(dist2):2:0 from the reciprocal crosses and so the data from these were pooled for the Control cross (Table 1, Figure 2A). The incidence of PatDp(dist2):2:0 generated using the T26H translocation was not significantly different from that found previously [24]. There was no significant difference in the incidence of PatDp(dist2):2:2 compared to PatDp(dist2):2:0, not in the incidence of PatDp(dist2):2:1 or PatDp(dist2):1:2, but the incidence of PatDp(dist2):1:1 at birth was significantly greater than PatDp(dist2):2:0 (P = 0.0394, Fisher’s exact test, two tailed) (Table 2).

It should be borne in mind that the incidence of PatDp(dist2):2:1, PatDp(dist2):1:1 and PatDp(dist2):2:1 is likely to be an underestimate because of recombination between the TH26 breakpoint on chromosome 2 and the Gnas cluster which lie 21cM apart (www.informatics.jax.org) (May, 2012). Thus in Cross 1, the cross to generate PatDp(dist2):2:1, recombinants PatDp(dist2):2:0 and PatDp(dist2):2:2 were found. In Cross 3, the cross to generate PatDp(dist2):1:1, recombinants PatDp(dist2):2:0 and PatDp(dist2):2:0 occurred. In Cross 4, the cross to generate PatDp(dist2):1:2, recombinants PatDp(dist2):2:2 arose. All recombinants in Cross 2, the cross to generate PatDp(dist2):2:2, will be identical and indistinguishable from non recombinant PatDp(dist2):2:2 (Table 2). In the absence of recombination 4 more PatDp(dist2):2:1 were expected in Cross 1, 6 more PatDp(dist2):1:1 in Cross 3 and 1 more PatDp(dist2):1:2 in Cross 4. Using the expected numbers of PatDps that would have occurred in the absence of recombination the incidence of PatDp(dist2):2:1 is also significantly greater than PatDp(dist2):2:0 (P = 0.0259, Fisher’s exact test, two tailed). Overall the results indicate that dosage of Gnasxl and Gnas can affect survival of PatDp(dist2) to birth.

Interestingly a recombinant PatDp(dist2):2:0 homozygous for A Nevps that arose in Cross 3 had a normal phenotype and survived for 6 months. This is probably because in such a mouse the expected levels of Gnasxl expression would be nearly 50% of the wild type level and this appears to be sufficient for viability.

In agreement with earlier work PatDp(dist2):2:0 generated using the T26H translocation only survived for a few days [24]. However there was some increase in survival of PatDp(dist2):2:1 compared to PatDp(dist2):2:0 with a couple of PatDp(dist2):2:1 reaching 13 days but none survived to weaning (Table 2). For PatDp(dist2):2:2 and PatDp(dist2):2:1 where the expressed doses of Gnas and Gnasxl are expected to be in balance and in PatDp(dist2):1:2 predicted to have one expressed dose of Gnasxl and two expressed doses of Gnas, there was a remarkable increase in survival with most PatDp(dist2):2:2, PatDp(dist2):1:1 and PatDp(dist2):1:2 reaching weaning (Table 2). After weaning the viability of many of these PatDp(dist2) was as good as their non PatDp(dist2) littermates. Thus for long term survival relative overexpression of Gnasxl is disadvantageous but overexpression of imprinted Gnas is not.

Neonatal Phenotype

At birth the PatDp(dist2):2:0 mice had the typical phenotype (Table 3). Thus, most were noted to be oedematous although oedema is much less marked in PatDp(dist2):2:0 than in the Oed mutant, a point mutation in Gnas exon 6 [18,25]. Most PatDp(dist2):2:0 were described as chunky and over 90% were observed to be hyperactive within 24 hours of birth. The hyperactivity is very distinctive. Whereas non PatDp mice move very little shortly after birth, PatDp(dist2):2:0 tend to be in motion most of the time, circling or crawling, often rapidly, with the forelimbs showing a paddling motion (paddle feet). They frequently fall on their backs and thrash around but are able to right themselves. The hyperactive behaviour becomes more extreme with the passing days (Video S1, S2). About half were noted to have a tail kink and paddle feet. The occurrence of each of these features is highly significant (P<0.0001, Fisher’s exact test, two tailed) for none of these features was observed in the 351 non duplication mice generated in the cross.

Of note, none of the PatDp mice with either one or two doses of Gnas, thus PatDp(dist2):2:1, PatDp(dist2):2:2, PatDp(dist2):1:1 or PatDp(dist2):1:2 were oedematous (Table 3). As oedema is known to be associated with loss of functional imprinted Gnas in the presence of a single expressed dose of Gnasxl [7,9,25,26] it can be concluded that the oedema seen in PatDp(dist2):2:0 is attributable to lack of expression of Gnas in tissues in which it is imprinted and not to overexpression of Gnasxl.

Even though oedema was absent in PatDp(dist2):2:1, a number were noted to be chunky (Table 3), indicating that oedema and a chunky appearance are probably two separate components of the short square body shape phenotype seen in PatDp(dist2):2:0 [1].

There was also a diminution in the number of PatDp(dist2):2:1, PatDp(dist2):2:2, PatDp(dist2):1:1 and PatDp(dist2):1:2 described as chunky compared to PatDp(dist2):2:0. In addition, fewer PatDp(dist2):2:2 and PatDp(dist2):1:1 were chunky compared to PatDp(dist2):2:1 (Table 3). Overall these results indicate that a double dose of expressed Gnasxl together with loss of expression of imprinted Gnas gives rise to this phenotype. However, if the double dose of Gnasxl is matched with a double dose of Gnas the chunky phenotype was not seen. For normality the expressed doses of Gnasxl and Gnas need to be balanced (Table 3).

Hyperactivity in PatDp(dist2):2:0 although manifested on the day of birth, becomes fully developed by the next day (Table 3). There was a decrease in the incidence of hyperactivity in PatDp(dist2):2:1, PatDp(dist2):2:2, PatDp(dist2):1:1 and PatDp(dist2):1:2 both on the day of birth and the following day. By day 2 almost all PatDp(dist2):2:0 were hyperactive but hyperactivity was virtually absent in PatDp(dist2):2:2, PatDp(dist2):1:1 and PatDp(dist2):1:2. A single PatDp(dist2):1:1 was found to be hyperactive on day two, but appeared normal by day four and this mouse was also classified as chunky. These findings were unexpected as Gnas and Gnasxl have the same predicted expressed dosage in PatDp(dist2):1:1 as in wild type. Loss of functional imprinted Gnas in the presence of a single expressed dose of Gnasxl is not associated with hyperactivity in the first few days of life [7,18] although tremor and ataxia starting at around one week of age have been observed in one Gnas knockout model [7]. On the other hand hyperactivity in the first few days has been observed in another mutant which like PatDp(dist2):2:0 has a double dose of Gnasxl and loss of Gnas in imprinted tissues [27]. Overall the results indicate that overexpression of Gnasxl combined with loss of imprinted Gnas expression is responsible for the hyperactivity phenotype.

The incidences of tail kink and paddle feet, both known features of PatDp(dist2):2:0, are not significantly different in PatDp(dist2):2:1
but are reduced in PatDp(dist2)2:2, PatDp(dist2)1:1 and PatDp(dist2)1:2. Furthermore, fewer PatDp(dist2)2:2 and PatDp(dist2)1:1 exhibited either tail kink and/or paddle feet than PatDp(dist2)2:1 (Table 3). Overall these results indicate that a double dose of expressed Gnasxl together with either loss of, or a single dose of imprinted Gnas gives rise to this phenotype. For normality the expressed doses of Gnasxl and Gnas need to be balanced (Table 3).

Thus oedema can be attributed to lack of expression of Gnas in tissues in which it is imprinted, chunkiness, hyperactivity, tail kink and paddle feet to overexpression of Gnasxl, combined with loss of imprinted Gnas expression.

Altered Metabolism in Neonatal PatDp(dist2)2:0 but not PatDp(dist2)2:2

Studies of metabolism were undertaken in PatDp(dist2)2:0 and and PatDp(dist2)2:2 and their nonduplication littermates. The nonduplication littermates of PatDp(dist2)2:0 are wild type for AEx1A whereas nonduplication littermates of PatDp(dist2)2:2 are heterozygous +/AEx1A and thus have biallelic Gnas expression. We tested neonates for glucose and key hormones involved in energy homeostasis (Figure 4). In PatDp(dist2)2:0 blood glucose was considerably reduced and accordingly, plasma insulin was also low compared to their nonduplication sibs. Furthermore, the plasma glucagon level, expected to be raised in response to hypoglycaemia, was under half that found in nonduplication +/+ sibs. Plasma noradrenaline levels, expected to be raised in response to hypoglycaemia were elevated by more than 60% in PatDp(dist2)2:0. In contrast PatDp(dist2)2:2 had normal levels of glucose, insulin, glucagon and noradrenaline. Thus there is an overall disruption in glucose metabolism in PatDp(dist2)2:0 attributable to overexpression of Gnasxl combined with loss of expression of Gnas. Loss of Gnasxl expression in Gnasxlp/+ pups also results in hypoglycaemia but, surprisingly no elevation of plasma noradrenaline in newborns [10]. In the current study plasma noradrenaline was measured in 2 and 3 day old pups (P2 and P3) and no statistically significant difference was found in Gnasxlp/- and wild type littermates. The combined data for P2 & P3 were: 6.57 ng/ml ±0.67 versus 8.85±1.50; WT versus Gnasxlp/-; mean±s.e.m; n = 14 WT and 9 Gnasxlp/-; P = 0.19).

In newborn +/AEx1A the levels of glucose, insulin and glucagon and noradrenaline were not significantly different from wild type indicating that overexpression of Gnas is not associated with altered glucose metabolism.

Both PatDp(dist2)2:2 and +/AEx1A Mice Show Postnatal Growth Retardation

Further studies from birth to adulthood of PatDp(dist2)2:2 and their nonduplication +/AEx1A sibs were carried out. In addition studies of +/AEx1A and their +/+ sibs from a cross of C3H/HeH females to +/AEx1A males were undertaken. For weight studies between birth and weaning the weights of both sexes were combined as no significant differences in the weights of males and females were found, and after weaning only males were used. Mice were weighed on the day of birth and weekly thereafter.

The weights are shown as a percentage of their wild type siblings (Figure 5). The +/AEx1A mice were lighter at birth, 90.1% (P = 0.021), of the weight of their wild type siblings, indicating prenatal growth retardation. Growth retardation became more pronounced during the first week of life but by weaning +/AEx1A had started to catch up so that by nine weeks their weight was no longer significantly different from wild type (Figure 5A). Similar findings of prenatal and preweaning growth retardation followed by catch up growth has been reported on paternal inheritance of the Ex1A-T-CON allele which also results in loss of imprinted expression of Gnas [28].

PatDp(dist2)2:2 were generated by Cross 2, T26H/++/+ X T26H+/AEx1A/++AEx1A so all the nonduplication sibs were heterozygous for AEx1A. PatDp(dist2)2:2 did not differ in weight from their nonduplication +/AEx1A sibs at birth or at one week of age indicating that PatDp(dist2)2:2 were also growth retarded and to the same extent as +/AEx1A. By two weeks of age PatDp(dist2)2:2 were even lighter in weight than +/AEx1A and continued to weigh less than +/AEx1A until 6 weeks but subsequently did not differ significantly in weight from +/AEx1A. Thus postnatal growth retardation in PatDp(dist2)2:2 peaked at three weeks and was followed by catch up growth (Figure 5B).

Thus loss of imprinting of Gnas in +/AEx1A results in growth retardation before weaning resulting in proportionately smaller mice followed by catch up growth in adulthood. PatDp(dist2)2:2 with predicted balanced expressed dosage of Gnas and Gnasxl show a more severe pre-adult growth retardation resulting in proportionately smaller mice followed by catch up growth by 10–11 weeks of age and overall increased weight thereafter.

Table 1. Crosses to generate PatDp(dist2).

| Cross | Cross | PatDp | Genotype of PatDp | Expected Ratio |
|-------|-------|-------|-------------------|---------------|
| Control cross | T26H/++/+ X T26H/++/+ | PatDp(dist2)2:0 | T26H/++/+ | 2:0 |
| Cross 1 | T26H/++/+ X T26H/++/+AEx1A | PatDp(dist2)2:1 | T26H/++/+AEx1A | 2:1 |
| Cross 2 | T26H/++/+ X T26H+/AEx1A/++AEx1A | PatDp(dist2)1:1 | T26H+/AEx1A/++AEx1A | 1:1 |
| Cross 3 | T26H/++/+ X T26H+/+ Nespas + | PatDp(dist2)2:2 | T26H+/+ Nespas + | 2:2 |
| Cross 4 | T26H/++/+ X T26H+/+AEx1A+/+ Nespas + | PatDp(dist2)1:2 | T26H+/+AEx1A+/+ Nespas + | 1:2 |

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At 10 weeks of age +/-ΔEx1A tended to show increased oxygen consumption and carbon dioxide output in comparison with wild-type littermates and these increases were statistically significant (P<0.03) by 21 weeks of age in +/-ΔEx1A mice indicative of a raised metabolic rate (Figure 6).

Discussion

The oedema in PatDp(dist2):2:0 is likely to be due to loss of expression of imprinted Gnas as oedema was not seen in PatDp(dist2):1 in which Gnas expression was restored to normal. This agrees with the results from other studies indicating that null or loss of function mutations of Gnas are associated with neonatal oedema [1,7,9,26,27,29] but neonatal oedema was not observed when imprinted Gnas expression was restored to normal [6,30]. Oedema in PatDp(dist2):2:0 is known to develop from 12.5 dpc and by late gestation PatDp(dist2):2:0 are severely oedematous and Oed even more so with oedema declining somewhat in both genetic conditions before birth [18,20]. The occurrence of oedema in utero could conceivably affect survival to birth and so the restoration of imprinted Gnas expression in PatDp(dist2):2:1 and PatDp(dist2):1:1 may account for their increased incidence at birth compared to PatDp(dist2):2:0.

The other neonatal phenotypes of PatDp(dist2), namely lethality, hyperactivity, padder feet, chunky appearance, tail kink, can be attributed to biallelic expression of Gnasxl combined with loss of imprinted Gnas expression. A considerable improvement in the incidence of newborns that were neither hyperactive nor chunky and some improvement in survival to seven days was found when biallelic expression of Gnasxl was combined with normal monoallelic expression of Gnas in PatDp(dist2):2:1. However, for full viability and loss of hyperactivity, biallelic expression of Gnasxl needs to be balanced with biallelic expression of imprinted Gnas or monoallelic expression of Gnasxl with monoallelic expression of Gnas. This need for balanced dosage is in accord with observations indicating that maternally expressed Gnas and paternally expressed Gnasxl act antagonistically [10].

Interestingly monoallelic expression of Gnasxl combined with biallelic and thus overexpression of imprinted Gnas, in either PatDp(dist2):1:2 or +/-ΔEx1A results in normal viability. Normal viability was also recently reported for the Ex1A-T-CON mutant in which Gnas is overexpressed [28]. Thus overexpression of Gnasxl has less drastic results on phenotype than overexpression of Gnasxl. But in the presence of two expressed copies of Gnasxl it is critical to have two expressed copies of Gnas in order to survive to weaning.

PatDp(dist2):2:0 are hyperactive and hypoglycaemic and both these phenotypes may contribute to their early death. Hyperactivity has also been reported for some Nesp55m neonates [27] which like PatDp(dist2):2:0 have two expressed doses of Gnasxl and no expressed doses of imprinted Gnas. The hyperactivity in PatDp(dist2):2:0 and Nesp55m may reflect a central neurological deficit. The finding of raised noradrenaline in PatDp(dist2):2:0 together with a recent report showing that Gnasxl is expressed in neonatal muscle [31] may well have relevance for the hyperactivity phenotype. Conversely the hypotonia and inactivity of Gnasxl KO pups might be at least partly due to the lack of XLZs in muscle.

PatDp(dist2):2:0 are hypoglycaemic with low insulin and raised noradrenaline. Nesp55m neonates are also hypoglycaemic and although poor feeding may well contribute to their hypoglycaemia they may, in addition, have a defect in glucose counterregulation [26]. The causes of the hypoglycaemia in PatDp(dist2):2:0 are not known, and even though they have a milk spot they could have a...
feeding impairment that could contribute to the hypoglycaemia. Also, in view of their low glucagon, PatDp(dist2)2:0 could have a defect in glucose counterregulation. In PatDp(dist2)2:2 with balanced expressed doses of both Gnasxl and Gnas, glucose metabolism is restored to normal, suggesting that both Gnasxl and imprinted Gnas have a role in neonatal glucose metabolism.

Both Gsα and XLαs have several variants. Thus C-terminally truncated neural specific forms GsαN1 and XLN1 of Gsα and XLαs respectively are known [32,33]. In addition XLαs has a second ORF that encodes a protein ALEX and then there is an N-terminally extended form of XLαs called XXLαs [34,35,36]. On comparison of the phenotypes seen in mouse models in which different transcripts have been inactivated it appears that XLαs/XXLαs play a part in growth and metabolism, ALEX may affect suckling but roles for XLN1 and GsαN1 have not been established yet [28,37]. Possibly the metabolic defects in PatDp(dist2)2:0 can be attributed in part at least to overexpression of XLαs/XXLαs but the contribution of the various Gnasxl proteins to other aspects of the PatDp(dist2)2:0 phenotype is as yet unknown.

Our data indicate that both Gnas and Gnasxl have roles in the phenotype of PatDp(dist2). Loss of the orthologous GNAS transcript in humans is associated with a number of endocrine and bone disorders including pseudohypoparathyroidism, Albright Hereditary Osteodystrophy and progressive osseous heteroplasia [38]. Pseudohypoparathyroidism type Ia (PHP-Ia) is due to maternal inheritance of an inactivating mutation in GNAS and pseudohypoparathyroidism type Ib is caused by defective imprinting at the cluster (reviewed in [37]). In both conditions there is resistance to parathyroid hormone but PHP-Ia is also associated with multiple hormone resistance and Albright hereditary osteodystrophy (AHO). Some patients with PHP-Ib have mild AHO features indicating overlap in the PHP-Ia and PHP-Ib phenotypes.

### Table 2. Incidence and Survival of PatDp(dist2).

| Cross          | PatDp                  | Expected Ratio | Incidence at birth | Survival to 7d | Survival to weaning |
|----------------|------------------------|----------------|-------------------|----------------|---------------------|
|                |                        | Gnasxl: Gnas   | No               | %              | No                 | %                  | No                 | %                  |
| Control cross  | PatDp(dist2)2:0        | 2:0            | 31/428           | 7              | 3/28               | 8                  | 0/28               | 0                  |
| Cross 1        | PatDp(dist2)2:1        | 2:1            | *18/171          | 10             | 5/14               | 36                 | 0/14               | 0                  |
| Cross 2        | PatDp(dist2)2:2        | 2:2            | 19/171           | 11             | 4/12               | 100                | 3/11               | 92                 |
| Cross 3        | PatDp(dist2)1:1        | 1:1            | ~31/258          | 12             | 3/19               | 90                 | 3/19               | 90                 |
| Cross 4        | PatDp(dist2)1:2        | 1:2            | 6/73             | 8              | 2/5/6              | 83                 | 3/5/6              | 83                 |

*23 PatDp altogether but 2 were PatDp(dist2)2:2 and 3 were PatDp(dist2)2:0.

*36 PatDp altogether but 3 were PatDp(dist2)0:2 and 2 were PatDp(dist2)2:0.

*10 PatDp altogether but 3 were PatDp(dist2)2:1 and one was not classified for dEx1A.

Incidence No shows the number of PatDps born/total number of mice born.

Survival No shows number of PatDps/total number of PatDps scored.

For comparison of PatDp(dist2)2:0 with other PatDp classes:

1P < 0.05, 2P < 0.01, 3P < 0.0001 (Fisher’s exact test, 2 tailed).

For comparison of PatDp(dist2)2:1 with other PatDp classes except PatDp(dist2)2:0:

1P < 0.002, 2P < 0.0001 (Fisher’s exact test, 2 tailed).

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### Table 3. Neonatal Phenotype of PatDp(dist2).

| PatDp            | Expected Ratio | Oedema | Chunky | Hyperactive | Tail kink | Paddle feet |
|------------------|----------------|--------|--------|-------------|-----------|-------------|
|                  | Gnasxl: Gnas   | No     | %      | No%         | No%       | No%         | No%          | No%         |
| PatDp(dist2)2:0  | 2:0            | 22/25  | 88     | 20/24       | 83        | 14/21       | 67           | 15/16       | 93          | 12/20       | 69          | 11/20       | 55          |
| PatDp(dist2)2:1  | 2:1            | *0/14  | 0      | ~5/14       | 36        | ~2/13       | 15           | *3/8        | 37          | 6/14        | 43          | 4/14        | 29          |
| PatDp(dist2)2:2  | 2:2            | *0/19  | 0      | z1/0/19     | 0         | *0/17       | 0            | *1/12       | 8           | *1/19       | 5           | *3/0/19     | 0           |
| PatDp(dist2)1:1  | 1:1            | *0/28  | 0      | z1/0/28     | 18        | *0/28       | 0            | *1/20       | 5           | *2/28       | 7           | *2/28       | 7           |
| PatDp(dist2)1:2  | 1:2            | *0/6   | 0      | ~1/6        | 17        | *0/6        | 0            | 0/6         | 0           | 0/6         | 0           | 0/6         | 0           |

For comparison of PatDp(dist2)2:0 with other PatDp classes:

1P < 0.001;

1P < 0.0001 (Fisher’s exact test, 2 tailed).

For comparison of PatDp(dist2)2:1 with other PatDp classes except PatDp(dist2)2:0:

1P < 0.01;

2P < 0.05 (Fisher’s exact test, 2 tailed).

No indicates number of PatDp with the phenotype/total number of PatDp classified.

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Figure 4. Metabolic analyses in early postnatal stages. (A) blood glucose levels. (n = 9–10) (B) plasma insulin levels. (n = 10–14) (C) plasma glucagon levels. (n = 9–13) (D) plasma noradrenaline. (n = 10–38). Error bars show standard errors of the means; ***P < 0.001, **P < 0.01, *P < 0.05. doi:10.1371/journal.pone.0065639.g004

Figure 5. Growth retardation. (A) Growth curve of +/ΔEx1A and wild-type littermates from 1 day to 12 weeks. The weights of wild-type littermates have been normalised to 1 at each timepoint and the weights of +/ΔEx1A mice have been taken as a percentage of wild-type weights. (n = 5–26). (B) Growth curve of PatDp(dist2)2:2 and +/ΔEx1A. The weights of +/ΔEx1A littermates have been normalised to 1 at each timepoint and the weights of PatDp(dist2)2:2 mice have been taken as a percentage of +Ex1A weights (n = 9–12). Error bars show standard errors of the means. doi:10.1371/journal.pone.0065639.g005
imprinted GNAS expression together with overexpression of GNASXL or microdeletions upstream of the cluster resulting in loss of imprinted GNAS expression alone [48,49,50,51]. Thus the clinical features of PHP-Ib appear to be due to loss of imprinted GNAS, and overexpression of GNASXL does not appear to play a significant role in the phenotype. However two of the patUPD20 patients showed neonatal hypoglycaemia [40,45] which, in view of the findings in PatDp(dist2:2:0) in the mouse, could be due to overexpression of GNASXL. In neonatal PatDp(dist2) mice, hyperactivity commencing shortly after birth and elevated noradrenaline are also features of raised Gnasxl levels. It would be of interest to ascertain if metabolism and activity in PHP-Ib patients are affected in the early postnatal period and if so, whether elevated GNASXL is responsible.

We have shown that loss of imprinting of Gnas in +ΔEx1A results in postnatal growth retardation followed by catch up growth and can be attributed solely to loss of imprinting, resulting in biallelic expression, of Gnas. The effects on postnatal growth in +ΔEx1A parallel the growth pattern seen in +/Ex1A-T-CON mice which also have loss of imprinting of Gnas [28]. Thus maternally expressed Gnas clearly has a role in growth. Paternally expressed Gnasxl is also known to affect postnatal growth and mutations resulting in loss of functional Gnasxl also result in postnatal growth retardation [7,10,11,18,28,29,52] The finding that both overexpression of Gnas and underexpression of Gnasxl results in growth retardation accords with the parental conflict theory that predicts that maternally expressed imprinted genes will be growth inhibiting and paternally expressed imprinted genes will be growth enhancing [12]. Thus it was expected that postnatal growth would be unaffected in PatDp(dist2:2:2) mice which have a predicted balanced dosage of Gnas and Gnasxl. However PatDp(dist2:2:2) mice show a more extreme postnatal growth retardation than seen with overexpression of Gnas in +ΔEx1A. Thus biallelic expression of both Gnas and Gnasxl has resulted in postnatal growth retardation. One interpretation is that it is important to have monoallelic expression of antagonistic Gnasxl and Gnas for normal growth. The advantages of monoallelic expression of both Gnasxl and Gnas must outweigh the costs associated with functional haploidy.

Supporting Information

Figure S1 Expression of Gnasxl was reduced on paternal inheritance of A Nespas. A Northern blot of Gnasxl and β-actin loading control using 2.5 μg of poly(A)+ RNA from 15.5-dpc embryos. MatDp(dist2) have no expressed copies of Gnasxl whilst PatDp(dist2) have two expressed copies, leading to the absence of the 2.5 kb band and the presence of a strong 2.5 kb band respectively. (B) Bar chart showing the Gnasxl expression levels in +/ΔEx1A (mean ± s.e.m 24±3.56%) were decreased on comparison to wild-type (+/+) (mean ± s.e.m 103±9.39%) *P = 0.005 (Student's t test, two-tailed). The mean ± s.e.m was calculated for 3 wild-type (+/+ ) and four +/ΔNespas.

Video S1 Hyperactivity in PatDp(dist2). Video of a 6 day old PatDp(dist2) and wild-type littermate showing almost continual activity and rapid movement with paddling of the front feet in the PatDp(dist2) but very little movement in the wild-type. The PatDp(dist2) has the characteristic tail kink or bend, and by 6 days is clearly smaller than the wild-type but oedema and a chunky appearance are no longer evident. The mice have a genetic background that is 50% Mus musculus castaneus and 50% laboratory mouse. On this background there was slightly better survival of PatDp(dist2) to 7 days (10/23) compared with 3/28 on an entirely...
laboratory mouse background (P = 0.0106 ( Fisher’s exact test, 2-tailed) but no difference in survival to weaning. (MP1)

**Video S2 Hyperactivity in PatDp(dist2)**. Video of the same PatDp(dist2) and wild-type littermate at 6 days as in Video S2 and the PatDp(dist2) at 5 days of age on its own. The video shows the ability of the PatDp(dist2) to right itself after falling on its back, as well as its continual activity and rapid movement. (MP1)

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**Author Contributions**

Conceived and designed the experiments: JP RDC. Performed the experiments: STB MLK JER MTD HJ LJ DN CVB TH AP BMC JP. Analyzed the data: STB MLK JER MTD TH AP BMC RDC JP. Wrote the paper: JP.
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