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Developmental programming of appetite and growth in male rats increases hypothalamic serotonin (5-HT)5A receptor expression and sensitivity

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Competing interests

The authors have no conflicts of interest.

Abstract

Background: Though it is well established that neonatal nutrition plays a major role in lifelong offspring health, the mechanisms underpinning this have not been well defined. Early postnatal accelerated growth resulting from maternal nutritional status is associated with increased appetite and body weight. Likewise, slow growth correlates with decreased appetite and body weight. Food consumption and food-seeking behaviour are directly modulated by central serotonergic (5-hydroxytryptamine, 5-HT) pathways. This study examined the effect of a rat maternal postnatal low protein (PLP) diet on 5-HT receptor mediated food intake in offspring.

Methods: Microarray analyses, in situ hybridization or laser capture microdissection of the ARC followed by RT-PCR were used to identify genes up- or down-regulated in the arcuate nucleus of the hypothalamus (ARC) of 3-month-old male PLP rats. Third ventricle cannulation was used to identify altered sensitivity to serotonin receptor agonists and antagonists with respect to food intake.

Results: Male PLP offspring consumed less food and had lower growth rates up to 3 months of age compared to Control offspring from dams fed a normal diet. 97 genes were upregulated (including the 5-HT_{5A} receptor (5-HT_{5A}R) and 149 downregulated genes in PLP rats compared to Controls. The former obesity medication fenfluramine and the 5-HT receptor agonist 5-Carboxamidotryptamine (5-CT) significantly suppressed food intake in both groups, but the PLP offspring were more sensitive to d-fenfluramine and 5-CT compared to Controls. The effect of 5-CT was antagonized by the 5-HT_{5A}R antagonist SB699551. 5-CT also reduced NPY-induced hyperphagia in both Control and PLP rats but was more effective in PLP offspring.

Conclusions: Postnatal low protein programming of growth in rats enhances the central effects of serotonin on appetite by increasing hypothalamic 5-HT_{5A}R expression and sensitivity. These

findings provide insight into the possible mechanisms through which a maternal low protein diet during lactation programs reduced growth and appetite in offspring.

Introduction

Developmental programming describes the growth and nutritional patterns during early life that influence the risk of developing disease in later life (Bianco-Miotto *et al*, 2017). Postnatal overnutrition and rapid postnatal growth during early life are associated with long-term susceptibility to obesity in both humans and animal models (Ozanne and Hales, 2004; Rkhzaf-Jaf *et al*, 2012). In contrast, slow growth during early postnatal life is linked to decreased obesity in later life (Jimenez-Chillaron *et al* 2006; Remmers *et al*, 2008; Stocker *et al*, 2012). Studies of short catastrophic famine conditions, such as the Dutch Hunger Winter and Chinese famines, provide natural experiments to directly examine the long-term health effects of profound nutritional changes at different stages of early life. Children, particularly males, born to mothers exposed to undernutrition *in utero* during early gestation showed an increased propensity to obesity; conversely, those exposed in the last trimester of pregnancy or in early postnatal life had a reduced risk of obesity as adults (Ravelli *et al*., 1976)).

Reducing growth and weight gain during lactation in rodents similarly results in a permanent reduction in body weight and food intake, greater leanness when fed a chow diet, resistance to diet-induced obesity as well as improved insulin and leptin sensitivity (Ozanne and Hales, 2004; Jiminez-Chilleron *et al*, 2006; Cripps *et al*, 2009). The precise molecular mechanisms linking altered nutrition in early life and long-term energy balance are still under investigation. However, programmed changes in the hypothalamus (Stocker *et al*, 2012; Wattez *et al*, 2013; Tungalagsuvd *et al*, 2016) and brown and white adipose tissue (Garcia *et al*, 2011; Claycombe *et al*, 2013, Palou *et al*, 2015) are implicated.

The hypothalamus differentiates *in utero*, but continued maturation occurs into early postnatal life in both rodents and humans (Grove *et al*, 2005; Glavas *et al*, 2007). Studies in animal

models have shown that during this period the expression of neuropeptides and their receptors can be permanently altered by the maternal diet (Muhlhausler *et al*, 2006; Delahaye *et al*, 2008; Chen *et al*, 2008). The arcuate nucleus of the hypothalamus (ARC) is a key homeostatic brain region modulating appetite and body weight (Heisler and Lam, 2017). It has been reported that maternal dietary modification can modulate central serotonergic pathways and feeding behaviour in rats (Paradis *et al*, 2017) and the 5-HT_{1A}R, 5-HT_{1B}R and 5-HT_{2A}R levels and receptor-mediated feeding behaviour are altered in rats exposed *in utero* to a maternal low protein diet (Lopes de Souza *et al*, 2008; Manuel-Apolinar *et al*, 2014; Martin-Gronert *et al*, 2016). 5-HT receptors have long been demonstrated to directly affect food consumption and food-seeking behaviour (Blundell *et al*, 1995; Calu *et al*, 2014; D'Agostino *et al*, 2018) and have been proposed as targets for anti-obesity therapies (Burke and Heisler, 2015). The aim of the current study was therefore to investigate the effect of a maternal low protein diet during the suckling period (undernutrition) on the mechanisms of 5-HT-mediated food intake.

Materials and Methods

Experimental groups and tissue collection

All procedures involving rats were conducted following approval by the University of Cambridge and the University of Buckingham Ethical Review Processes and in accordance with project licences under the British Home Office Animals (Scientific Procedures) Act (1986). The breeding of Wistar rats (Charles River, Ltd, Margate, United Kingdom) was conducted at both the University of Cambridge (for laser-capture microdissection (LCM) and the University of Buckingham (for for intracerebroventricular (i3v) studies). Detailed information regarding the diet composition and the set-up of the maternal protein restricted (8% protein) and Control dams have been published previously (Petry *et al*, 1997; Cripps *et al*, 2009; Stocker *et al*, 2012). The day after birth (P1) two experimental groups of offspring were

established: Controls [offspring of Control dams, culled to 8 (four males and four females) suckled by Control dams], and postnatal low protein [PLP; offspring of dams (4 males and 4 females) fed Control diet during pregnancy, but nursed by low protein dams]. The body weight of the pups was recorded at P1, P7, P14 and P21. Following weaning at day 21, all male pups were fed standard laboratory chow and body weight and food intake recorded weekly. One male pup per litter was culled at P7, 14 and 22 for serum and their hypothalami dissected. At 3 months of age the remaining males were culled, and their brains collected. All the dissected brains and hypothalami were frozen on powdered dry ice and were stored at - 80°C until further processing.

Laser Capture Microdissection (LCM) and RNA isolation

Hypothalamic sections of the arcuate nucleus (n=6 Control and n=6 PLP each from a different litter) were prepared as described previously (Martin-Gronert *et al*, 2016). Specifically, the ARC was sectioned on a cryostat at 14µm thickness from approximately -4.52 to -2.30 mm relative to the bregma (Paxinos and Watson, 1998). Sections were collected onto RNase-free membrane-coated slides (P.A.L.M) that had been baked at 200ºC for 4 hours and UV cross-linked for 30 minutes. On average 10 sections were collected per slide (in one movement) and 18-20 slides were obtained per brain. Within 24 hours of sectioning, sections were placed for 30 seconds each time in 95% ethanol, and then in 75% and 50% ethanol for rehydration. Sections were stained with 1% cresyl violet stain (Ambion, Foster City, California, USA) for 1 minute, dehydrated in graded ethanol concentrations (50%, 75% and twice in 100% for 30 s each time), HistoClear (National Diagnostics (Atlanta, Georgia, USA) for 5 minutes and air dried. LCM was performed using a P.A.L.M. MicrolaserSystem (P.A.L.M. Microlaser Technologies, Burkhardtsdorf, Germany). Following microdissection, the captured cells were kept in RNA*later* (Ambion). Total RNA was isolated from LCM samples using the

RNAqueous Micro RNA extraction kit (Ambion) in accordance with the manufacturer's protocol. The quality and quantity of the RNA samples was determined using the Agilent BioAnalyzer PicoChips (Agilent Technologies Inc, Santa Clara, California, USA).

RNA amplification

Ovation Pico RNA Amplification System (Nugen Technologies Inc, San Carlos, California, USA) was used for the amplification of RNA destined for microarray analysis. RNA amplification of LCM ARC samples (n=6 Control and n=6 PLP each from a different litter) used to validate genes identified by microarray analysis was performed using a MegaScript T7 Amplification Kit (Ambion) in combination with the GeneChip sample CleanUp Module kit (Affymetrix Inc, Santa Clara, California, USA). The use of a different method of RNA amplification enhanced the validation of the microarray data.

Microarray hybridization

The amplified RNA was used for gene expression profiling on Affymetrix Rat Genome 230 2.0 Arrays (Affymetrix Inc) using the Affymetrix GeneChip protocol to fragment and label the target, ready for hybridization to the arrays. GeneChip sequences were selected from GenBank, dbEST and RefSeq and the sequence clusters created using UniGene were then further refined by comparison with the publicly available assembly of the rat genome. Microarray hybridization was carried out by Molecular Biology Services at University of Warwick, using n=6 chips per group (each from a different litter). The Control array data is the same as that used in our previous paper and the PLP array was run in parallel (Martin-Gronert *et al*., 2016).

Microarray analysis and selection of the genes for validation

Raw image data files were converted to *CEL* and pivot files using Affymetrix GeneChip Operating Software (GCOS). All downstream analysis of microarray data was performed using

GeneSpring GX 12.0 (Agilent). The *CEL* files were used for the Robust Multi-array Averaging (RMA) (Irizzarry *et al*, 2003) and GeneChip RMA (GC-RMA) (Wu *et al*, 2004) analyses, while the pivot files were used for GCOS analysis. After importing the data (n=6 Control and n=6 PLP each from a different litter), each chip was normalized to the 50^{th} centile of the measurement taken from that chip and all gene expression data reported as a fold-change from the control state. Genes were considered to be up- or downregulated if the genes had differential expression of $P<0.05$ in comparison to the Control group, if 1.3-fold threshold was reached (statistical criterion described previously in Martin-Gronert *et al*, 2016). Only genes that met the above criteria using GCOS, RMA and GCRMA were taken forward for additional study. The further selection of genes for validation was based on the function of the gene and the availability of suitable primers for validation. Potential consequences of gene expression dysregulation were gained from Ingenuity Pathway Analysis (Ingenuity Systems Inc.). Data have been deposited in Gene Expression Omnibus (accession number GSE76012) at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76012.

Validation of microarray data using Taqman RT-PCR

Validation of the microarray data was carried out using Micro Fluidic Cards (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's protocol (Applied Biosystems). The reactions were performed in duplicate for each sample using an ABI 7900HT (Applied Biosystems). A standard curve was constructed for each gene using a serial dilution of pooled cDNA from all LCM ARC samples. The mean C_T values of the experimental samples were then used to calculate the relative expression for each sample and data were normalized to *Ppia (cyclophilin)*, expression of which did not change between postnatal maternal treatment groups. RNA from the hypothalamus of 3-month-old rats was used for gene expression analyses with GAPDH as an internal control. Real time PCR (StepOne^{TM}, Applied Biosystems)

was carried out using Assay on Demand pre-designed primer and probe sets (Applied Biosystems) (n=8 control and n=8 PLP). Data were analysed using the comparative ΔC_t method, comparing PLP rats with Controls. All procedures were carried out in accordance to the manufacturer's recommendation and the genes assayed are listed in Table 1.

Effects of centrally administered compounds on food intake

Male and female rats (n=21 Control and n=29 PLP) were cannulated as described previously (Stocker *et al*, 2012). A cannula was inserted into the third ventricle under gaseous anaesthetic (isoflurane: Isoba, Schering-Plough Animal Health, Kenilworth, New Jersey, USA) using coordinates from the stereotactic rat brain atlas (Paxinos and Watson, 1998). Its position was verified by a positive drinking response over 15 minutes to angiotensin II (50 ng in 2.5 µl). For measurements of acute effects on food intake of d-fenfluramine, 5-HT_{5A}R agonism, 5-HT_{2C}R agonism alone or in combination with a selective 5-HT_{5A}R antagonist, if administered the antagonist, it was dosed 15 minutes prior to the agonist. 3-month-old rats were individually housed, fasted for 4 hours prior to the onset of dark, dosed at the beginning of the dark period, food returned, and intake recorded 1, 2 and 4 hours post-dosing. A similar procedure was followed for NPY and 5-HT_{5A}R agonist combination studies, except that NPY was administered 30 minutes after the dose of the 5-HT_{5A}R agonist at the beginning of the light cycle. Rats were dosed using a within-subjects procedure with a Latin square design to identify drug dose treatment order. Doses were separated by at least 4 days and normal feeding behaviour and body weight was restored prior to administration of the next dose. The specificity and doses of d-fenfluramine, the combination of 5-HT_1R/5-HT_5R/5-HT_7R agonist 5-carboxamidotryptamine maleate (5-CT) and selective 5-HT_{5A}R antagonist SB699551, the high affinity 5-HT_{2C}R agonist CP 809101 hydrochloride (2-[(3-Chlorophenyl)methoxy]-6-(1-piperazinyl)pyrazine hydrochloride) (Tocris Biosciences, Bristol, UK) and NPY (Bachem, St

206 Helens, UK) were based on published data (Muñoz-Islas *et al*, 2014; Siuciak *et al* 2007;
207 Jourdan *et al*, 2003; Nikiforuk *et al* 2016; Martin-Gronert *et al* 2016; Stocker *et al*., 2012).
208 Drug was delivered in 2.5 µl saline or DMSO as indicated.

209

210 ***Statistical analysis***

211 Student's *t*-test was used for statistical analysis unless otherwise stated. A priori power analysis
212 was conducted using G*Power3 (Faul *et al*, 2007). Fractional growth rates were calculated
213 using the formula: fractional growth rate = (current - starting weight)/ (period x starting
214 weight). Total Quantitative Real-Time PCR gene expression data was normalized to *Ppia*. One-
215 and two-way ANOVA was used for the analysis of the food intake data. All data sets passed
216 the Anderon-Darling test for normality of distribution (alpha of 0.05). Outliers as identified by
217 ROUT were excluded (Motulsky and Brown, 2006). All litters were represented in the results.
218 Offspring from each litter was selected randomly. Animals were randomly allocated by the
219 Project Licensee, not the principle investigator. The data are presented as mean ± SEM values
220 unless otherwise stated. $P<0.05$ was used to signify statistical significance. Vairiances were
221 similar between all statistically compared groups. The study has been perfomed once.

222

223 **Results**

224 ***PLP rats are hypophagic and have reduced body weight***

225 PLP rat pups were smaller than Controls by day 7 (9.2 ± 0.2g versus 16.1 ± 0.3g; $P<0.0001$)
226 and remained smaller throughout lactation, weighing 66% less than Controls at day 21 (17.4g
227 ± 0.4g versus 51.8 ± 1.0g; $P<0.0001$) (Figure 1A). At 3 months of age, PLP offspring
228 consumed less food (23.1 ± 1.2 $g.animal^{-1}.day^{-1}$ vs. 29.5 ± 1.6 $g.animal^{-1}.day^{-1}$; $P<0.001$),
229 gained less weight ($P<0.001$; Figure 1B) and had a lower body weight compared to Controls

(*P*<0.001; Figure 1B). At the age of 22 days there was no difference in the absolute brain weight between the PLP rats and Controls (Figure 1B. However, the weight of the brain was significantly higher in PLPs as a proportion of body weight (*P*<0.001) indicating that the growth of the brain was spared (Figure 1B). The sparing of the brain in the PLP rats was also apparent at three months of age (*P*<0.001; Figure 1B**)**.

Genes upregulated and downregulated in the ARC of PLP rats

ARC microarray data were analysed using three different methods: GCOS, GC-RMA and RMA (Figure 2). The three analyses revealed that 97 genes were upregulated, and 149 genes were downregulated in the ARC of PLP rats compared to Controls. The top 25 upregulated (Table 2) and downregulated genes (Table 3) were ranked according to fold change. Of the upregulated genes, 11 are involved in neuronal proliferation, regeneration, development, differentiation or remodeling. 12 are responsible for generic neuronal or epididymal structural or molecular function. 2 of the upregulated genes are directly involved in neurotransmission: *Grm7*, a glutamate receptor involved in most aspects of brain function; and *Htr5a*, the 5-hydroxytryptamine receptor 5A. Of the downregulated genes, 9 are repressors of cell growth and differentiation or inducers of neural degeneration/apoptosis. 11 are responsible for generic neuronal or epididymal structure or molecular function, including modulators of the blood brain barrier. *Plau*, *Cry2* and *Serpinb1a* are components circadian rhythm. *Igsf11* is an immunoglobulin highly expressed in the brain. Angptl4 (an LPL inhibitor in the periphery) is centrally expressed mainly in the glial cells and influences central control of whole-body metabolism. Angptl4 has been positively correlated with obesity and the metabolic syndrome, and both type 1 and type 2 diabetes (Vienberg *et al*, 2014).

Of these, the eleven genes previously reported to be associated with central regulation of adiposity and nutritional energy balance were selected to be validated by real-time PCR. The

two genes identified by transcriptional profiling as upregulated in PLP rats selected to be validated by PCR were the serotonin 5A receptor *Htr5a* (5-$HT_{5A}R$; $P<0.05$) and the rho guanine nucleotide exchange factor *Kalrn* ($P<0.05$) (Figure 3). Out of the genes that were predicted to be downregulated in the ARC of the PLP group, nine were selected for validation. Out of these, three genes were expressed at very low levels, below the threshold of sensitivity of our assay (*Plau*, *Gbp2* and *Cp*). One gene (*Cry2*) was not different between groups as measured by PCR (100 ± 16.1 for Controls versus 110.1 ± 18.3 (% mean Control) for PLP). *Rest* was one of the top genes downregulated in PLP rats (Figure 3). *Cdk5R1* (*Cdk5/p35*), *Dok1*, *Txnip* were also significantly downregulated. RT-PCR confirmed that all these transcripts were reduced in the PLP rats in comparison to the Controls including *Rest* ($P=0.07$), *Cdk5R1* ($P<0.01$), *Dok1* ($P<0.05$) and *Txnip* ($P<0.05$). The overall validation rate by PCR was 86%.

PLP rats are more sensitive to 5-HT obesity medication d-fenfluramine

A key effect of maternal PLP is lifelong reduced food intake and body weight in offspring. 5-HT is an established neurotransmitter modulating feeding and body weight. Taking advantage of this biological effect, medications augmenting 5-HT bioavailability have been used to affect human feeding and body weight over the past few decades, such as the global obesity medication d-fenfluramine (Burke and Heisler, 2015).

To investigate whether changes in 5-HT circuitry might underpin PLP rats reduced feeding behaviour and body weight, we began by measuring hypothalamic 5-HT levels. No differences in hypothalamic 5-HT was detected between Control (1354+86 $pmol.g^{-1}$; n = 12) and PLP (1415+139 $pmnol.g^{-1}$; n = 12) offspring. We next stimulated the release of endogenous 5-HT and blocked its reuptake with d-fenfluramine. D-fenfluramine was administered directly into

the third ventricle and food intake recorded in 3-month-old PLP and Control rats. D-fenfluramine significantly suppressed food intake in both Control and PLP rats (Figure 4). However, PLP rats were significantly more sensitive to d-fenfluramine compared to Controls (P<0.05) 2 hours post dosing. These data suggest that PLP may impact 5-HT brain circuitry modulating energy homeostasis.

PLP rats are more sensitive to the anorectic effect of 5-CT

5-HT_{2C}R is the primary 5-HT receptor in the ARC that mediates both 5-HT and d-fenfluramine-induced reduction in food consumption (Heisler *et al.*, 2002)." (Heisler et al., Science 2002). However, we detected no differences in ARC 5-HT_{2C}R expression in PLP compared to Control rats, suggesting that action at this receptor is unlikely to explain the differences in sensitivity to d-fenfluramine. We investigated a functional difference in 5-HT_{2C}R agonist responsivity by administering CP 809101 or vehicle into the third ventricle and measuring food intake. No overall significant difference was detected in the feeding response to CP 809101 between the Control and PLP groups when compared by 2-way ANOVA (Figure 5A-B). These findings illustrate that PLP rats are not broadly more sensitivity to compounds that reduce feeding.

D-fenfluramine will increase endogenous 5-HT activity at all receptors and we found that ARC 5-HT_{5A}R expression is increased with PLP diet. Like d-fenfluramine, partial 5-HT_{5A}R agonist 5-CT suppresses food intake (Martin-Gronert *et al*, 2016). To investigate how changes in 5-HT_{5A}R gene expression in PLP rats might influence food intake, we administered 5-CT into the third ventricle and measured its effects on food intake. Similar to d-fenfluramine, PLP offspring were more sensitive to the anorectic effect of 5-CT compared to Controls one hour (P<0.01; Figure 5C) and two hours after dosing (P<0.05; Figure 5D). To confirm the specificity

at the 5-HT_{5A}R we performed a combination study with 5-CT and a specific 5-HT_{5A}R antagonist SB699551. SB699551 (30 nmol) significantly blocked the effect of a half-maximal dose of 5-CT of 5 nmol in PLP and 10 nmol in Control offspring (Figure 5E-F). These data suggest that the upregulation of ARC 5-HT_{5A}Rs in PLP rats increases sensitivity to the feeding effect of 5-HT_{5A}R agonism.

5-CT reduces the orexigenic effect of Neuropeptide Y

NPY is a potent stimulator of hunger and one of the brain regions of NPY expression is the ARC. To investigate specifically how the changes in 5-HT_{5A}R gene expression might influence central mechanisms of food intake, we administered the partial 5-HT_{5A}R agonist 5-CT directly into the third ventricle and measured its effects on NPY-induced food intake in adult 3-month-old offspring. A half-maximal dose of NPY was used based on previous studies (Stocker *et al*, 2012) and this was confirmed here. 5-CT reduced the NPY-induced food consumption in both Control and PLP groups but was more effective in the postnatal low protein offspring (Figure 6).

Discussion

The quality of postnatal diet has a lifelong impact on offspring health, appetite and body weight. To gain insight into the mechanisms underpinning maternal diet programming of dysregulated appetite and body weight, here we examined a key homeostatic brain region essential for the normal regulation of feeding behaviour, the ARC. ARC microarray identified the 5-HT system as one of the most affected targets in PLP offspring. 5-HT has been implicated in programming and regulation of hyperphagia and obesity in rat and mice offspring from dams undernourished during pregnancy (Lopes de Souza *et al*, 2008; Martin-Gronert *et al*, 2016; Manuel-Apolinar *et al*, 2014). Conversely, rats from mothers fed low protein diet during the

postnatal suckling period exhibit hypophagia and resistance to obesogenic diets and this study investigated this in relative to the 5-HT system. The maternal 8% protein diet during lactation reduced offspring body weight, particularly during the suckling period. However, other than being smaller there were no adverse effects associated with this severe malnutrition such as increased mortality. This corresponds to previous reports that show 8% protein diet-fed dams displayed lactational deficiency resulting in reduced postnatal growth and modulated peripheral metabolism, but no other overt symptoms of malnutrition as generated by lower protein levels (Resnick *et al*, 1982; Miller *et al*, 1980; Gabr, 1981). Resnick (Resnick et al, 1982) concluded that the 8% protein model is useful for studying "hidden" forms of malnutrition in man (Resnick *et al*., 1982). The lack of data in female offspring is a limitation of this study given the growing recognition of sex differences in programming (Dearden et al., 2018) and future studies should incorporate this factor in their design.

Relative to body weight, food intake in the PLP offspring was 7.53% of body weight compared with 7.07% in the controls. However, fat has less metabolic activity than lean tissue; possibly as little as one sixth relative to weight (Arch and Trayhurn, 2013), so the food intake of the PLP offspring may be what would be predicted from their body weight and composition. Unfortunately, we do not have body composition data, which is a limitation of the study.

This raises the cause-and-effect relationship between the effects of the PLP diet on food intake and on body weight and composition. If the PLP diet drives changes in body weight and composition by reducing long-term food intake, it is to be expected that energy intake will stabilise at a level that is very close to that predicted from body weight and composition. Obese and lean subjects fall on the same regression lines that link energy expenditure to body weight and composition, and only small imbalances in energy intake and energy expenditure are

355 required for obesity (or leanness) to develop over time (Arch and Trayhurn, 2013). An
356 alternative perspective is that the primary effect of the PLP diet may be on body weight and
357 composition, with food intake per mouse being reduced, by the mechanisms elucidated in the
358 present study, to accommodate the reduced metabolic demands of the PLP offspring. Others
359 have similarly raised the cause-and-effect relationship between overeating and obesity
360 (Ludwig and Friedman, 2014).
361
362 Of the seven 5-HT receptor families and fourteen receptor subtypes, 5-HT_2 is the predominant
363 family associated with appetite, particularly 5-HT_{2C}R (Heisler *et al*, 2002; Lam *et al*, 2010).
364 Additionally, 5-HT_{1A}R, 5-HT_{1B}R and 5-HT_4R agonism have been associated with decreased
365 appetite (Kumar *et al*, 2010; Jean *et al*, 2012) and 5-HT_6R associated with overeating (Pratt *et*
366 *al*, 2009). 5-HT_{5A}Rs are implicated in psychiatric behaviour (Kassai *et al*, 2012), have been
367 associated with plasma triglyceride availability (Zhang *et al*, 2010) and food-seeking initiation
368 (Pickens *et al*, 2012). Recently it has been discovered that maternal dietary modification can
369 cause profound changes within the rat hypothalamus that affect food consumption (Stocker *et*
370 *al*, 2012; Wattez *et al*, 2013; Tungalagsuvd *et al*, 2016) and 5-HT receptors, among other
371 factors, have been implicated in these changes (Paradis *et al*, 2017).
372
373 In this study, 5-HT_{5A}R agonism with 5-CT significantly suppresses food intake in Control rats
374 and in PLP offspring. The relative effect was greater in PLP offspring than in Control rats with
375 the effect being completely reversed by the 5-HT_{5A}R antagonist, SB699551. Whilst 5-CT has
376 highest affinity with the rat 5-HT_{5A}R it also agonizes 5-HT_1R and 5-HT_7R families (Nelson,
377 2004). However, the lack of overlap in activity of 5-CT and the specific 5-HT_{5A}R antagonist,
378 SB699551 (Muñoz-Islas *et al*, 2014), coupled with the gene expression differences suggests
379 that it is the 5-HT_{5A}R that is responsible for the differences observed in 5-HT-mediated

inhibition of food consumption. The lack of a demonstrable difference between the groups in 5-HT_{2C}R action is consistent with the suggestion that it is the 5-HT_{5A}R that is the main subtype affected in the offspring of dams fed a low protein diet during the postnatal period.

In addition to the 5-HT_{5A}R gene expression upregulation we identified several genes involved in homeostasis and neuronal development in the ARC of PLP rats that were permanently expressed at a lower level because of maternal protein restriction during lactation. *Rest* is a transcriptional repressor that is critical for neurogenesis and neuronal differentiation and plasticity (Chen *et al*., 1998). REST has been found to induce de-repression of the 5-HT_{1A}R gene (Lemonde *et al*, 2004), although its influence on other 5-HT receptor subtypes has yet to be investigated. *Cdk5R1* (*Cdk5/p35*) is a key regulator of neuronal cytoskeleton and an important determinant of neuronal death/survival signals (Dhariwala and Rajadhyaksha, 2008) and has been previously associated with a preference for high calorie food intake in mice exposed *in utero* to a maternal high fat diet (Teegarden *et al*, 2009). *Dok1* plays a role in immunoreceptor signalling (Mashima *et al*., 2009) and has been implicated in the development of obesity (Hosooka *et al*., 2008). *Txnip* is an essential component of a redox signalling pathway, through which it mediates oxidative stress responses (Schulze *et al*., 2004) and cell proliferation (Rani *et al*., 2010). Txnip null mice are resistant to obesity-associated insulin resistance (Chutkow *et al*, 2010). It has also been linked to growth, development and differentiation of the brain, central leptin sensitivity, regulation of energy balance and the development of diabetes (Levendusky *et al*, 2009; Chen *et al*, 2008; Lappalainen *et al*., 2009; Blouet *et al*., 2012) and may contribute to lower body weight, resistance to diet-induced obesity and improved glucose tolerance in PLP offspring (Cripps *et al*., 2009; Ozanne *et al*, 2004).

In conclusion, maternal low protein diet during the suckling period was associated with of 97 upregulated and 149 downregulated genes in the homeostatic brain region the ARC in PLP rats compared to Controls. The ARC is an essential regulator of appetite and PLP mice exhibit lifelong reduced feeding and body weight. Of particular interest was an upregulation of 5-HT_{5A}Rs because increases in 5-HT bioavailability is established to decrease feeding rodents and humans. PLP offspring were also more sensitive to the anorectic effect of endogenous 5-HT, as stimulated by d-fenfluramine and 5-HT_{5A}R agonism. These data suggest that PLP programs a greater number of ARC 5-HT_{5A}Rs which are positioned to respond to 5-HT's regulation of feeding behaviour. This may contribute to the effect of PLP to maintain reduced food intake and body weight throughout adulthood.

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644 **Figure Legends**

645

646 **Figure 1. Growth trajectory and brain weights of Control and PLP rats**. ***A***, Growth
647 trajectory throughout the suckling period illustrates lower body weight in postnatal low protein
648 (PLP) rats when compared to age-matched Controls. ***B***, Body weight and brain weights at
649 postnatal day 22 (P22), 3-11 weeks and 3 months (3M) of age in PLP and Control rats
650 illustrated reduced body weight, but not brain weight, in PLP rats. Statistical analysis by 2-way
651 ANOVA followed by Sidak's multiple comparisons test (n =10 per group). ★★★★
652 $P<0.0001$,***$P < 0.001$. Values are expressed as means ± S.E.M.

653

654 **Figure 2. Venn diagrams of maternal protein restriction during lactation on ARC gene**
655 **expression in 3-month-old male offspring according to three different analyses: GCOS,**
656 **GC-RMA and RMA.** 97 genes were upregulated, and 149 genes were downregulated in
657 postnatal low protein (PLP) rats when compared to Controls (C). The sizes of circles and
658 numbers in parentheses indicate the number of genes as identified by either the GCOS, RMA
659 or GC-RMA algorithms.

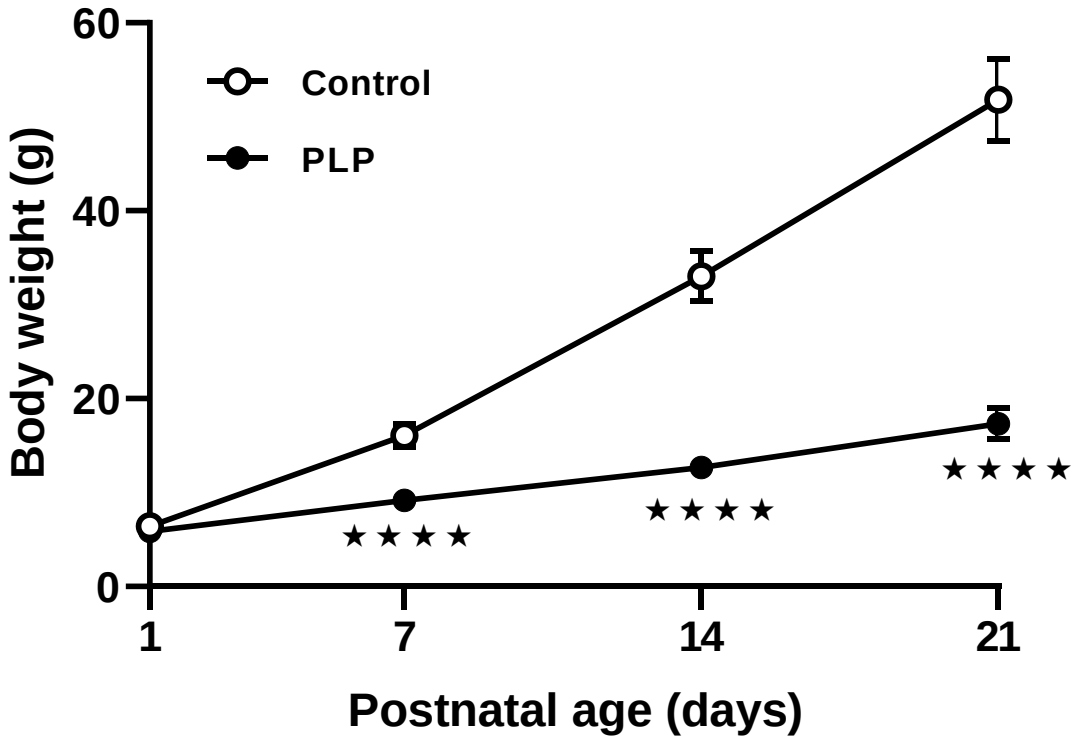
660

661 **Figure 3. RT-PCR validation of the differentially expressed genes in the ARC of Control**
662 **and postnatal low protein (PLP) rats identified with microarray**. Analysis carried out
663 using RT-PCR. Gene expression was normalized to housekeeping Control *Ppia.* Values are
664 expressed as means ± S.E.M. (n=8 per group). Data were analysed using a one-tailed
665 unpaired Student's t-test. * $P<0.05$; ** $P<0.01$. Values are expressed as means ± S.E.M.

666 **Figure 4. PLP rats are more sensitive to the anorectic effect of d-fenfluramine**. Food intake
667 was measured 2 hours after third ventricle administration of 250 nmol d-fenfluramine (DEX)
668 or vehicle (VEH) to 3-month-old Control (n=20) and postnatal low protein (PLP; n=20) rats.

669 ★P<0.05; ★★P<0.01; ★★★★P<0.0001, for food intake differences as a percentage of
670 saline, between Control and PLP rats. Values are expressed as means ± S.E.M.
671
672 **Figure 5. Enhanced 5-HT-induced food intake in PLP rats is 5-HT$_{5A}$R-mediated.** Food
673 intake was measured ***A***, 1 and ***B***, 2 hours after administering the 5-HT$_{2C}$R agonist CP 809101
674 to 3-month-old Control and postnatal low protein (PLP) rats (n=17 to 18 per group). Food
675 intake was measured ***C***, 1 and ***D***, 2 hours after administering the 5-HT$_{5A}$R agonist 5-CT to 3-
676 month-old Control and PLP rats (n=12 to 16 per group). Statistical analysis of each time
677 point was by 2-way *ANOVA* followed by Sidak's multiple comparisons test, ★★ P<0.01,
678 ★★★ P<0.001. Food intake following central administration of a submaximal dose of the 5-
679 HT$_{5A}$R agonist 5-CT and a selective 5-HT$_{5A}$R antagonist SB699551 was measured ***E***, 1 and
680 ***F***, 2 hours after administering 5-CT with or without 30 nmol SB699551 to 3-month-old
681 Control and PLP rats (n=11 to 16 per group). ★P<0.05; ★★P<0.01; ★★★ P<0.001. Values
682 are expressed as means ± S.E.M.Values are expressed as means ± S.E.M.
683
684 **Figure 6. Effect of 5-HT$_{5A}$R agonist 5-CT combined with NPY on food intake**. Food intake
685 was measured 2 hours after administering 5-CT with the half-maximal dose of NPY to 3-
686 month-old Control (n=21) and PLP rats (n=20 to 29 per group). ★★★P<0.001. Values are
687 expressed as means ± S.E.M.
688
689 **Table 1. Genes assayed by PCR.**
690
691 **Table 2. Top 25 genes increased in the ARC of PLP offspring compared to Controls.**
692 **Table 3. Top 25 genes decreased in the ARC of PLP offspring compared to Controls.**
693

A



B

	Control	PLP
Body weight (g) at 3M	417 ± 9.0	306.6 ± 11.1 ***
Body weight gain (g) 3-11 wks	377.3 ± 5.8	297.4 ± 6.8 ***
Brain weight (g) at P22	1.44 ± 0.2	1.20 ± 0.2
Brain weight (% of BW) at P22	2.69 ± 0.23	6.00 ± 0.25 ***
Brain weight (g) at 3M	2.00 ± 0.3	1.78 ± 0.3
Brain weight (% of BW) at 3M	0.48 ± 0.01	0.59 ± 0.02 ***

Figure 1. Growth trajectory and brain weights of Control and PLP rats.

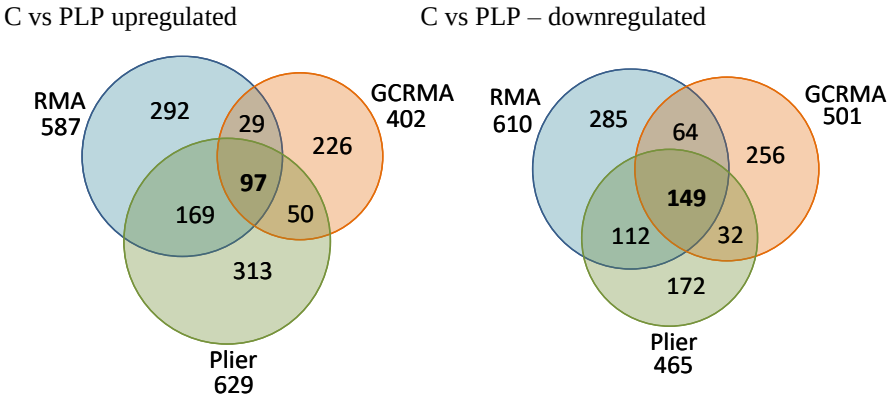


Figure 2. Venn diagrams of maternal protein restriction during lactation on ARC gene expression in 3-month-old male offspring according to three different analyses: GCOS, GC-RMA and RMA.

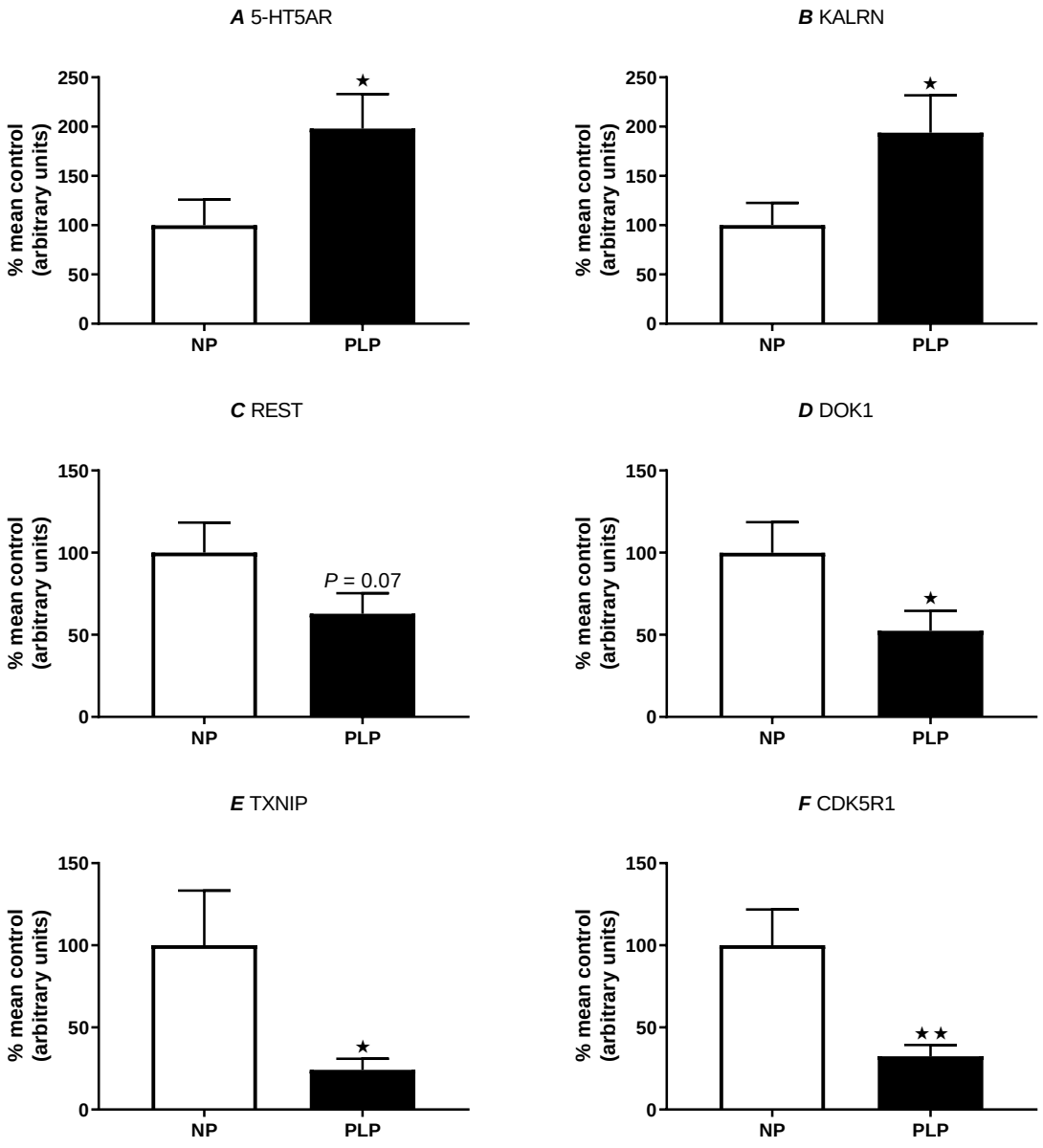


Figure 3. RT-PCR validation of the differentially expressed genes in the ARC of Control and postnatal low protein (PLP) rats identified with microarray.

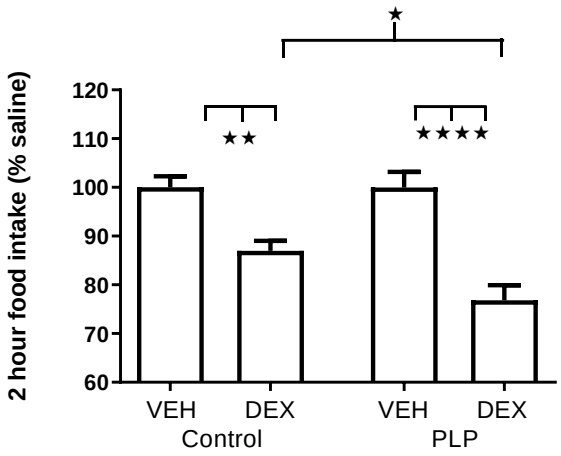


Figure 4. PLP rats are more sensitive to the anorectic effect of d-fenfluramine.

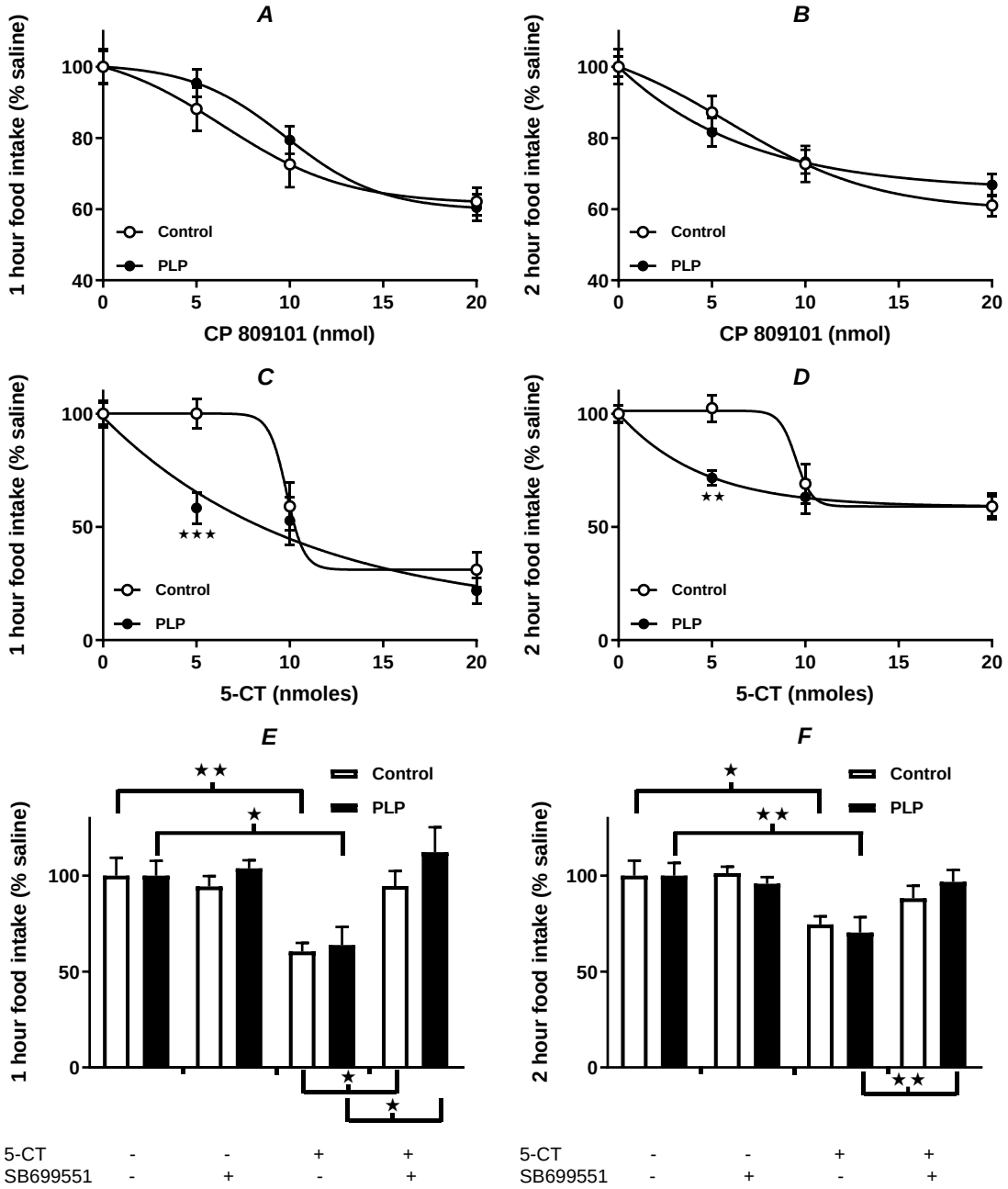


Figure 5. Enhanced 5-HT-induced food intake in PLP rats is 5-HT$_{5A}$R-mediated.

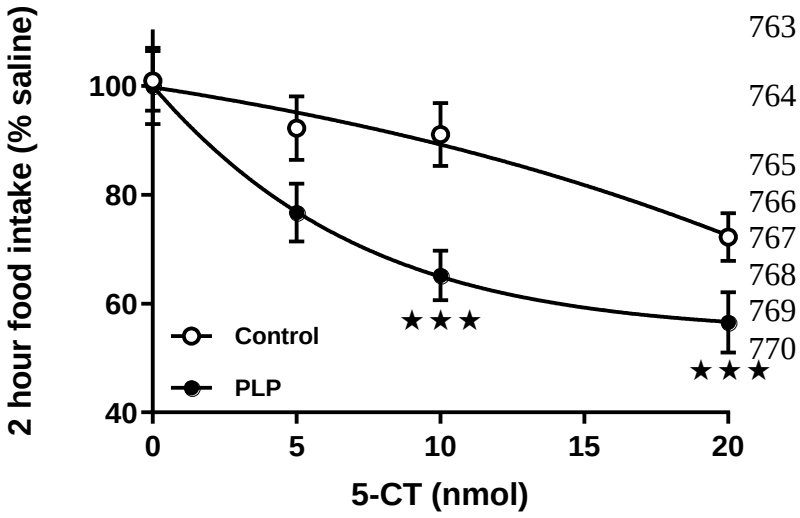


Figure 6. Effect of 5-$HT_{5A}R$ agonist 5-CT combined with NPY on food intake.

771 **Table 1. Genes assayed by PCR.**

Gene	Applied biosystems ID
AGRP	**Rn01431703_g1**
SF1	**Rn01450960_m1**
KALRN	**Rn00583225_m1**
REST	**Rn01413148_m1**
PLAU	**Rn00695755_m1**
HTR5A	**Rn00565746_m1**
HTR2A	**Rn00568473_m1**
GBP2	**Rn00592467_m1**
CRY2	**Rn00591457_m1**
KHSRP	**Rn00592338_m1**
CP	**Rn00561049_m1**
CDK5R1	**Rn02132948_s1**
HTR7	**Rn00576048_m1**
DOK1	**Rn01420942_g1**
SOCS3	**Rn00585674_s1**
GRP	**Rn00592059_m1**
HSDL1	**Rn01762355_m1**
IDH1	**Rn00580421_m1**
PFKFB2	**Rn00589696_m1**
STX6	**Rn00581473_m1**
TRHR2	**Rn00710465_m1**
POMC	**Rn00595020_m1**
NPY2R	**Rn00576733**
NPY1R	**Rn02769337_s1**
NPY5R	**Rn02089867_s1**
NPY	**Rn01410145_m1**
CARTPPT	**Rn01645174_m1**
INSR	**Rn00567070_m1**
IRS1	**Rn02132493_s1**
IRS2	**Rn01482270_s1**
AKT1	**Rn00583646_m1**
RETSAT	**Rn00595391_m1**
PDE4A	**Rn00565354_m1**
CAR8	**Rn01473820_m1**
TXNIP	**Rn0133885_g1**
EEF2K	**Rn00564087_m1**
BARHL1	**Rn00589045_m1**
BDNF	**Rn02531967_s1**
CCKBR	**Rn00565867_m1**
FOXM1	**Rn00581221_m1**
GAPDH	**Rn99999916_s1**
PPIA	**Rn00690933_m1**
POLR2A	**Rn01752026_m1**
POU3F3	**Rn02533545_s1**
LEPR	**Rn00561465_m1**
STAT3	**Rn00562562_m1**
JAK2	**Rn00580452_m1**
CCL6	**Rn01456402_g1**

772

773 **Table 2. Top 25 genes increased in the ARC of PLP offspring compared to Controls.**

Probe Set ID	Fold Change	Gene Symbol	Gene Title
1397955_at	**1.90**	**Rtel1**	regulator of telomere elongation helicase 1
1379905_at	**1.80**	**Gtpbp1**	GTP binding protein 1
1379128_at	**1.79**	**Arhgef17**	Rho guanine nucleotide exchange factor (GEF) 17
1370643_at	**1.77**	**Kalrn**	kalirin, RhoGEF kinase
1384502_at	**1.63**	**Fbxo42**	F-box protein 42
1382037_at	**1.47**	**Crim1**	cysteine rich transmembrane BMP regulator 1 (chordin like)
1369781_at	**1.45**	**Grm7**	glutamate receptor, metabotropic 7
1378424_at	**1.44**	**Trim46**	tripartite motif-containing 46
1373677_at	**1.43**	**Slc39a10**	solute carrier family 39 (zinc transporter), member 10
1391208_at	**1.42**	**Pcdh20**	protocadherin 20
1387850_at	**1.41**	**Tmeff1**	transmembrane protein with EGF-like & two follistatin-like domains 1
1385801_at	**1.40**	**Dnajc18**	DnaJ (Hsp40) homolog, subfamily C, member 18
1391600_at	**1.38**	**Mga**	MAX gene associated
1371027_at	**1.37**	**Cblb**	Cas-Br-M (murine) ecotropic retroviral transforming sequence b
1378269_at	**1.37**	**Dnalc1**	dynein, axonemal, light chain 1
1398125_at	**1.37**	**Ank2**	ankyrin 2, neuronal
1369463_at	**1.36**	**Htr5a**	5-hydroxytryptamine (serotonin) receptor 5A
1394599_at	**1.36**	**Zxdc**	ZXD family zinc finger C
1386049_at	**1.36**	**Kctd4**	potassium channel tetramerisation domain containing 4
1377714_at	**1.35**	**Pvrl3**	Poliovirus receptor-related 3
1397224_at	**1.35**	**Atp2b1**	ATPase, Ca++ transporting, plasma membrane 1
1387920_at	**1.35**	**Man2c1**	mannosidase, alpha, class 2C, member 1
1381205_at	**1.35**	**Snapc5**	Small nuclear RNA activating complex, polypeptide 5
1392942_at	**1.35**	**RGD1563325**	similar to hypothetical protein MGC17943
1368497_at	**1.34**	**Abcc2**	ATP-binding cassette, sub-family C (CFTR/MRP), member 2

774

775 **Table 3. Top 25 genes decreased in the ARC of PLP offspring compared to Controls.**

Probe Set ID	Fold Change	Gene Symbol	Gene Title
1387675_ at	**2.05**	**Plau**	plasminogen activator, urokinase
1388086_ at	**1.91**	**Rest**	RE1-silencing transcription factor
1373006_at	**1.86**	**Tmem171**	transmembrane protein 171
1369056_at	**1.84**	**Rpe65**	retinal pigment epithelium 65
1398621_at	**1.82**	**Ak7**	adenylate kinase 7
1368332_at	**1.79**	**Gbp2**	guanylate binding protein 2
1369446_at	**1.75**	**Cry2**	cryptochrome 2 (photolyase-like)
1388924_at	**1.72**	**Angptl4**	angiopoietin-like 4
1395184_at	**1.71**	**Clec12a**	C-type lectin domain family 12, member A
1368028_at	**1.71**	**Prph**	peripherin
1389123_at	**1.69**	**Ccl6**	chemokine (C-C motif) ligand 6
1377034_at	**1.68**	**Serpinb1a**	serine (or cysteine) proteinase inhibitor, clade B, member 1a
1382284_at	**1.66**	**Nek3**	NIMA (never in mitosis gene a)-related kinase 3
1388762_at	**1.65**	**Iqgap1**	IQ motif containing GTPase activating protein 1
1368418_ at	**1.65**	**Cp**	ceruloplasmin
1379068_at	**1.63**	**Armc9**	armadillo repeat containing 9
1397424_at	**1.62**	**Synpo2**	Synaptopodin 2
1385292_at	**1.62**	**Sc65**	Synaptonemal complex protein SC65
1381979_at	**1.60**	**Sumf2**	sulfatase modifying factor 2
1369538_at	**1.60**	**Cdk5r1**	cyclin-dependent kinase 5, regulatory subunit 1 (p35)
1371447_at	**1.59**	**Plac8**	placenta-specific 8
1391776_at	**1.57**	**RGD1305283**	COX assembly mitochondrial protein homolog (S. cerevisiae)
1372254_at	**1.56**	**Serping1**	serine (or cysteine) peptidase inhibitor, clade G, member 1
1376733_at	**1.56**	**Igsf11**	immunoglobulin superfamily, member 11
1384292_at	**1.47**	**Dok1**	docking protein 1
1371131_at	**1.46**	**Txnip**	thioredoxin interacting protein

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