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5-HT_{2A} and 5-HT_{2C} receptors as hypothalamic targets of developmental programming in male rats

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List of abbreviations

5-HT	5-hydroxytryptamine, serotonin
5-HT_{2A}R	5-hydroxytryptamine 2A receptor
5-HT_{2C}R	5-hydroxytryptamine 2C receptor
α-MSH	α-melanocyte-stimulating hormone
ARC	Arcuate nucleus of the hypothalamus
CEL	Cell intensity files
E16.5	Embryonic day 16.5
FFT	Free fraction of tryptophan
GCOS	GeneChip operating software
GC-RMA	GeneChip robust multi-array averaging
IUGR	Intra-uterine growth restricted
ISHH	*In situ* hybridization histochemistry
LCM	Laser captured micro dissection
POMC	Pro-opiomelanocortin
P3	Postnatal day 3
RMA	Robust multi-array averaging
TCB2	[(4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine hydrobromide
VMN	The ventromedial nucleus of the hypothalamus

Summary Statement

Maternal protein restriction increases fetal brain 5-HT and may contribute to changes in expression and function of hypothalamic 5-HT_{2C} and 5-HT_{2A} receptors in the offspring later in life.

Translational Impact

Clinical issue:

As the prevalence of obesity increases across the globe major efforts are directed at studying the mechanisms involved. Growing evidence suggests that susceptibility to obesity can be programmed by maternal and neonatal nutrition. In particular, individuals with low birth weight that undergo rapid postnatal growth are at higher risk of developing increased adiposity in later life. The molecular mechanisms mediating the interaction between early nutrition and obesity risk are, however, still largely unknown. Currently, there are no pharmacological therapies to prevent the increase in adiposity in individuals that underwent nutritional challenge in early life. Serotonin (5-HT) system is widely recognized as one of the key regulators of food intake and body weight. During fetal life serotonin also acts as a trophic factor that regulates brain development. As compounds targeting endogenous serotonin bioavailability have been among the most clinically effective treatments for obesity (e.g. d-fenfluramine), treatment targeting serotonin system may prevent the development of obesity in at risk individuals.

Results:

The study has shown that maternal protein restriction increased levels of serotonin in the blood of pregnant rat mums and in the placenta and the fetal brain. In adulthood the offspring had a reduced ability to sense serotonin, which was associated with a reduced hypothalamic expression of 5-HT_{2C} receptors - the primary serotonin receptor influencing appetite. As expected, reduced 5-HT_{2C} receptor expression was associated with impaired sensitivity to serotonin-mediated appetite suppression and hence could lead to increases in food intake. On the other hand offspring of low protein dams had a programmed increase in 5-HT_{2A} receptors, which are positioned, just like 5-HT_{2C} receptors, in the arcuate nucleus of the hypothalamus - a key area of the brain, involved in the control of food intake. Moreover, these animals were more sensitive to 5-HT_{2A} receptor agonist-induced appetite suppression.

Implications and future directions:

This study identified a molecular mechanism that is involved in mediating the effects of suboptimal maternal diet on the regulation of food intake in the offspring. In addition, the authors showed that a treatment with 5-HT_{2A} receptor agonist may potentially prevent the development of obesity in individuals that were exposed *in utero* to suboptimal nutrition and then underwent rapid postnatal growth. Non-hallucinogenic 5-HT_{2A} receptor agonist should be used for such treatment. Combining the 5-HT_{2A} receptor agonist with 5-HT_{2C}R and 5-HT_{1B}R agonists could potentially further increase the anorectic therapeutic effect.

Abstract

Though obesity is a global epidemic, the physiological mechanisms involved are little understood. Recent advances reveal that susceptibility to obesity can be programmed by maternal and neonatal nutrition. Specifically, a maternal low protein diet during pregnancy causes decreased intrauterine growth, rapid postnatal catch-up growth and increased risk for diet-induced obesity. Given that the synthesis of the neurotransmitter 5-hydroxytryptamine (5-HT) is nutritionally regulated and 5-HT is a trophic factor, we hypothesized that maternal diet influences fetal 5-HT exposure, which then influences central appetite network development and the subsequent efficacy of 5-HT to control energy balance in later life. Consistent with our hypothesis, pregnant low protein fed rat mothers exhibited elevated serum 5-HT, which was also evident in the placenta and fetal brains at E16.5. This increase was associated with a reduced hypothalamic expression of the primary 5-HT receptor influencing appetite, 5-HT_{2C}R in the fetal brain as well as neonatal and adult hypothalamus. As expected, reduced 5-HT_{2C}R expression was associated with impaired sensitivity to 5-HT-mediated appetite suppression in adulthood. 5-HT primarily achieves effects on appetite via 5-HT_{2C}R stimulation of pro-opiomelanocortin (POMC) peptides within the arcuate nucleus of the hypothalamus (ARC). We reveal that 5-HT_{2A}Rs are also anatomically positioned to influence the activity of ARC POMC and that 5-HT_{2A}R mRNA is increased in the hypothalamus of *in utero* growth restricted offspring that underwent rapid postnatal catch-up growth. Furthermore, these animals at 3-months of age are more sensitive to 5-HT_{2A}R agonist-induced appetite suppression. These findings may not only reveal a 5-HT-mediated mechanism underlying programming of obesity susceptibility but also provide a promising means to correct it, via a 5-HT_{2A}R agonist treatment.

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Introduction

Nutrition during critical periods of development in early life can exert profound, long-term effects on susceptibility to obesity. For example, men exposed to the Dutch Hunger Winter *in utero* during early gestation had an increased risk of developing obesity as adults, while obesity rates were reduced amongst those exposed to famine during the last trimester of gestation and in early postnatal life (Ravelli et al., 1976). The early postnatal diet is also important. In randomized trials, full-term, low birth weight infants fed a growth-promoting nutrient-enriched formula had a higher fat mass at the age of 5-8 years than those fed standard formula (Singhal et al., 2010). Studies in rodents, showing that low birth weight followed by rapid postnatal growth is associated with increased adiposity, support findings in humans (Plagemann et al., 1992; Cottrell et al., 2011; Berends et al., 2013). Despite these robust associations, the molecular mechanisms mediating the interaction between early nutrition and obesity risk are still largely unknown.

It is widely acknowledged that the hypothalamus, which in humans develops primarily prenatally but in rodents postnatally, plays an important role in the programming of body weight (Grove et al., 2005; Horvath and Bruning, 2006; Glavas et al., 2007). To date major efforts have been directed at understanding the roles of leptin and insulin in this process (Bouret et al, 2010; Yura et al., 2005; Steculorum and Bouret, 2011). However, studies in our laboratory using the leptin-deficient *ob*/*ob* mice demonstrated that leptin-independent mechanisms are likely to also program body weight (Cottrell et al., 2011).

The importance of the 5-hydroxytryptamine (5-HT; serotonin) system in the control of food intake and body weight has been recognised for many years and alterations in central serotonergic activity have been observed in obese humans, non-human primates and rodents (Mori et al., 1999; De Fanti et al., 2001; Sullivan et al., 2010). 5-HT, a potent anorectic signal, influences food intake in the mature brain by acting predominantly via 5-HT_{2C}R to regulate the key energy balance mediator, pro-opiomelanocortin (POMC), within the arcuate nucleus of the hypothalamus (ARC) (Doslikova et al., 2013; Burke et al., 2014). Indeed, genetic disruption of the 5-HT_{2C}R specifically expressed on POMC neurons programs hyperphagia and obesity when animals are fed a high-fat diet (Berglund et al., 2013). In addition to playing an important role in energy balance and body weight, 5-HT acts as a trophic factor during fetal brain development in rodents, regulating cell migration, proliferation, maturation and axonal growth (Whitaker-Azmitia et al., 1996; Gaspar et al., 2003). The long term effects of disturbances in the 5-HT system during early life on obesity risk are, however, not fully defined.

Here we investigated 5-HT system function and used an unbiased genome-wide profiling approach to identify differentially expressed targets in the hypothalamus of intrauterine-restricted rats that underwent postnatal catch-up growth (recuperated animals) in an effort to elucidate the molecular mechanisms mediating the effects of early nutrition on energy balance.

Results

Growth trajectories, body weights and brain weights

At birth, offspring of mothers fed a low protein diet during pregnancy were significantly smaller than the control pups ($P<0.001$) (Table 1A). The body weight of the recuperated pups caught up with that of control pups by day 14 and they weighed the same as controls at weaning (Table 1A). Fractional growth rate did not differ between recuperated and control pups between birth and day 7 of life (0.16 ± 0.1 vs 0.17 ± 0.01); however recuperated offspring had significantly higher fractional growth rate between birth and days 14 and 21 when compared to controls (day 14: 0.31 ± 0.01 vs 0.24 ± 0.01, $P<0.001$; day 21: 0.38 ± 0.01 vs 0.29 ± 0.01, $P<0.001$). At 3 months of age body weights were similar between recuperated and control offspring. Brain weights were similar between the experimental animals and their age-matched controls at both 22 days and 3 months of age, in both absolute terms and relative to body weight (Table 1B). These findings demonstrate that maternal low protein diet decreases *in utero* growth of the offspring so that they have a lower birth weight. These animals then undergo "catch up" growth such that by 22 days of age, they are the same body weight as the control offspring.

Maternal and offspring 5-HT and tryptophan levels

Maternal, placental and amniotic fluid 5-HT and tryptophan levels

Tryptophan is an essential amino acid and is required for 5-HT synthesis. A low protein diet would therefore be expected to yield low plasma tryptophan levels. We analyzed the levels of 5-HT and tryptophan in the dams, placenta and amniotic fluid to investigate whether 5-HT may be a programming factor that mediates changes in appetite and susceptibility to diet-induced obesity in recuperated animals. As expected, we observed reduced levels of tryptophan in the serum of low protein fed dams at E16.5 of pregnancy compared to control dams (158 ± 17 $mmol.l^{-1}$ vs 220 ± 20 $mmol.l^{-1}$, $P<0.05$). Paradoxically, the serum 5-HT level was raised in low protein diet fed dams at this time-point ($P<0.05$; Figure 1A). A similar pattern was observed for tryptophan and 5-HT concentration in in the amniotic fluid at E16.5, with tryptophan levels being reduced in the amniotic fluid from low protein pregnancies (26.3 ± 3.2 $mmol.l^{-1}$ vs 40.6 ± 3.4 $mmol.l^{-1}$, $P<0.01$, n=10-11 per group), whilst 5-HT levels were raised (65.0 ± 6.6 $nmol.l^{-1}$ vs 48.2 ± 3.9 $nmol.l^{-1}$, $P<0.05$, n=10-11 per group). 5-HT levels were also significantly increased in E16.5 placentas from low protein pregnancies when compared to control pregnancies ($P<0.01$; Figure 1A). Furthermore, we observed a negative correlation between placental 5-HT level and maternal serum tryptophan ($P<0.05$; Figure 1B). There was a positive correlation between placental 5-HT and fetal brain 5-HT levels (Figure 1C). These findings reveal that in response to significantly reduced dietary tryptophan, 5-HT is over-produced in the mother and in the intrauterine environment in which the fetus grows and develops.

Fetal, Neonatal, Weaning and Adult tryptophan and 5-HT levels

Likewise, in the neonatal brain, a decrease in tryptophan levels (67.9 ± 13.6 mmol.l^{-1} vs 140 ± 14.7 mmol.l^{-1}, *P*<0.01) was associated with an increase in 5-HT levels in the hypothalami of recuperated pups at birth (*P*<0.01; Figure 1A). 5-HT levels were also increased in the brains of E16.5 fetuses from low protein pregnancies (Figure 1A). By weaning as well as at 3-months of age, hypothalamic 5-HT levels were normalized to control levels (P21: Recuperated 1347 ± 110 mmol.l^{-1} vs Control 1659 ± 131 pmol.g^{-1} n= 12 per group; 3 months of age: Recuperated 1414 ± 154 mmol.l^{-1} vs Control 1354 ± 85 pmol.g^{-1} n= 10 per group). These data indicate that the over-production of 5-HT in response to maternal low protein diet is normalized by weaning.

Effect of D-fenfluramine on food intake

We next assessed the significance of maternal diet-stimulated increased 5-HT during early development on the function of the adult 5-HT system. D-fenfluramine is a pharmacological probe of the endogenous 5-HT system that works by stimulating endogenous 5-HT release and blocking its reuptake. D-fenfluramine was prescribed until the 1990s for human obesity treatment since increasing endogenous 5-HT bioavailability reduces appetite and body weight (Lam et al., 2010; Marston et al., 2011). We administered D-fenfluramine into the third ventricle of rats at 3 months of age and observed the expected reduction in food intake in controls (Figure 2). However, recuperated offspring had impaired sensitivity to 5-HT-stimulated (D-fenfluramine) food intake reduction (*P*<0.05) (Figure 2). These results show that a low protein maternal diet followed by accelerated postnatal growth diminishes 5-HT mediated control of food intake in the adult offspring. Given the strong inverse correlation between 5-HT and appetite, these findings lead us to hypothesise that deregulated 5-HT system may contribute to the programmed increased food intake in these offspring when exposed to an obesogenic diet.

Fetal, neonatal and postnatal hypothalamic 5-HT$_{2C}$R expression

5-HT is a neurotransmitter that communicates appetite-related signals primarily through the 5-HT$_{2C}$R within the ARC. If 5-HT's effects on appetite are diminished in recuperated rats, this suggests a perturbation in signalling at the 5-HT$_{2C}$R. We next probed the consequence of elevated 5-HT during development on the expression of the 5-HT$_{2C}$R. Analysis of E16.5 fetal brain *5-HT$_{2C}$R* mRNA expression showed significantly reduced *5-HT$_{2C}$R* in the whole heads of fetuses from low protein pregnancies (*P*<0.05; Figure 3A). At birth *5-HT$_{2C}$R* was still reduced in the hypothalamus of the growth restricted pups at the mRNA (*P*<0.001; Figure 3A) as well as at the protein (*P*<0.001; Figure 3B,C) level. However, by 3 months of age no significant differences in *5-HT$_{2C}$R* mRNA levels were observed between the experimental groups (Figure 3A). Protein expression was, however, still significantly reduced in the hypothalamus of recuperated animals at 3-months of age (*P*<0.05; Figure

3B,C). Given that perturbations of ARC 5-HT_{2C}R function increases appetite and body weight (Berglund et al., 2013), these results thereby reveal a potential mechanism through which rapid postnatal growth is achieved in recuperated rats, which also could potentially contribute to increased susceptibility to diet-induced obesity in these animals.

Microarray analysis of LCM ARC from control and recuperated 3-month-old offspring

5-HT is a trophic factor during fetal brain development influencing both 5-HT circuits and the anatomical organisation of other systems (Whitaker-Azmitia et al., 1996; Gaspar et al., 2003). The ARC is a crucial regulator of energy balance. Having demonstrated that maternal diet induces changes in offspring 5-HT and disrupts 5-HT appetite system function, we next probed whether 5-HT disrupts other ARC genes and circuits regulating appetite. To achieve this, we performed LCM of the ARC of 3-month-old control and recuperated rats and subjected samples to microarray.

Validation of laser-capture micro dissection (LCM)

To ensure the specificity of the ARC dissection within the LCM material (Figure 4), we analyzed the expression of two genes that are not expressed in the ARC but are expressed in the neighbouring hypothalamic nuclei. Neither PVN-specific *Brn1* (*Pou3f3*) nor the ventromedial nucleus of the hypothalamus (VMN) specific *Sf-1* were detected (data not shown). In addition, we examined the expression of four key genes involved in the regulation of energy balance in the ARC: *POMC, AgRP, CART* and *NPY*. We confirmed using Taqman RT-PCR that all of these genes were highly expressed in the dissected material (data not shown).

Analysis of microarrays using three different algorithms and pathway analysis

Following global transcriptional profiling, we analyzed the data using three different algorithms (1) GeneChip Operating Software (GCOS), (2) Robust Multi-array Averaging (RMA) and (3) GeneChip Robust Multi-array Averaging (GCRMA) to ensure maximum stringency and to reduce the number of false positives. The combined analysis, using a 1.3- fold cut off threshold and $P<0.05$, revealed that out of 31,099 genes analyzed, expression of 15951 genes was detected in the ARC. Of these 142 genes were up-regulated in the ARC of the recuperated animals, while 59 genes were down-regulated (Figure 4A and 4B respectively). The top 25 up- and down-regulated genes in recuperated offspring are presented in Tables 2A and 2B. *Htr2a,* which codes for *5-HT_{2A}R,* was the gene with the highest (2.55-fold) increase. We further analyzed the microarray data using Ingenuity Pathway Analysis software to detect groups of functionally-related genes. The top three gene functions affected by early nutrition in 3-month-old male recuperated rats were cell cycle, connective tissue development and function, cellular growth and proliferation (Table 2C).

Validation of genes identified by microarray

Out of the seven genes chosen for validation, two genes had very low expression (*Plau* and *Pde4a*). Out of five genes remaining, differential expression of three genes (*5-HT$_{2A}$R*, *Khsrp* and *Retsat*) was confirmed by RT-PCR, whilst two genes were not validated (*Car8* and *EEF2k*) (Figure 5A). As the *5-HT$_{2A}$R* had the highest fold change in the microarray analysis (Table 2A), it was further investigated. The up-regulation of the *5-HT$_{2A}$R* in the ARC of 3-month-old recuperated animals (P<0.05) observed using RT-PCR was confirmed by *in situ* hybridization (P<0.05; Figure 5B).

Timing and location of increased hypothalamic 5-HT$_{2A}$R expression

A similar pattern of *5-HT$_{2A}$R* expression to that seen in 3-month-old animals was observed at the age of 22 days. *5-HT$_{2A}$R* was significantly elevated in the ARC of recuperated rats at weaning (P<0.05; Figure 5C). The expression of *5-HT$_{2A}$R* was, however, not altered in neonatal ARC (Figure 5C) or in E16.5 fetal brains (Low protein 0.933±0.035; Control 1.002±0.022). As in the ARC the expression of *5-HT$_{2A}$R* was also significantly increased in VMN of 3-month-old recuperated animals (P<0.05; Figure 5 D,E). However, in contrast to the ARC, no difference was seen in the *5-HT$_{2A}$R* expression within VMN between the two experimental groups at day 22 of life (Figure 5E) suggesting that up-regulation of *5-HT$_{2A}$R* within the VMN in the recuperated group is secondary to the up-regulation of this receptor in the ARC. Overall these findings reveal a change in 5-HT$_{2A}$R receptor expression using an unbiased genome-wide profiling approach and thereby support the importance of maternal diet induced changes in the 5-HT system within the hypothalamus in disrupted offspring energy balance.

Neuronal co-localization of ARC 5-HT$_{2A}$R and POMC

Within the ARC, 5-HT primarily influences energy balance via action at 5-HT$_{2C}$R stimulating the activity of POMC neurons (Heisler et al., 2002; Xu et al., 2008; Berglund et al., 2013; Burke et al., 2014). Given that *5-HT$_{2C}$R* expression is reduced in rats exposed to a maternal low protein diet, we hypothesized that *5-HT$_{2A}$R* may therefore be up-regulated to compensate for this in an effort to normalise energy balance. To determine whether 5-HT$_{2A}$Rs are anatomically positioned to influence the activity of POMC neurons, we visualized co-expression using dual histochemical labelling. We determined that approximately 40% of the ARC POMC neurons express *5-HT$_{2A}$Rs* in control rats (Figure 5F). These data reveal a defined population of neurons within the ARC critically involved in body weight regulation that could be influenced by G_q-protein coupled 5-HT$_{2A}$Rs.

Effect of 5-HT$_{2A}$R agonism on food intake

To assess the functional implications of upregulated ARC *5-HT$_{2A}$R* expression, we administered the selective 5-HT$_{2A}$R agonist TCB2 [(4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine hydrobromide] directly into the third ventricle and measured its effects on food intake

in adult 3-month-old recuperated and control offspring. We confirmed previous results showing that 5-HT_{2A}R agonism significantly suppresses food intake (Fox et al., 2010). TCB2 also significantly decreased food intake in the recuperated offspring as shown by two-way ANOVA analysis (overall effect of TCB2 administration, $P<0.001$; Figure 6). However, the dose response curves between the control and recuperated groups were significantly different ($P<0.05$; Figure 6). Recuperated offspring were more sensitive than the controls to the centrally administered action of 5-HT_{2A}R agonist TCB-2 (overall effect of early nutrition, $P<0.05$; Figure 6). These results reveal that over-expressed 5-HT_{2A}Rs in recuperated rats are functional and when pharmacologically stimulated, a greater net effect on appetite is achieved. Extrapolating these findings to endogenous 5-HT activity, as indicated by the D-fenfluramine results, suggests that the up-regulation of 5-HT_{2A}R is insufficient to compensate for 5-HT_{2C}R down-regulation in the 5-HT regulation of appetite.

4. Discussion

Evidence from epidemiological studies and animal models shows that suboptimal early nutrition affects susceptibility to obesity in later life. We and others have shown that rodents exposed to maternal protein restriction *in utero* and accelerated postnatal growth (recuperated offspring) are more susceptible to development of diet-induced obesity (Ozanne et al., 2004; Bieswal et al., 2006). In the current study we focused on 3-month-old recuperated rats fed a chow diet that are a similar weight to control offspring. This enabled us to investigate mechanisms mediating the effects of early nutrition on susceptibility to developing diet-induced obesity without the confounding effects of obesity itself. In recent years, leptin has been studied as a key factor involved in programming of obesity risk (Cripps et al., 2009; Bouret, 2004). However, we previously showed that programming of increased adiposity is, at least in part, leptin-independent (Cottrell et al., 2011). In the current study, we investigated the effect of a maternal low protein diet during gestation on a factor directly impacted by reduced dietary protein, 5-HT. We observed that 5-HT levels were increased in the fetal brains and neonatal hypothalami of rats exposed to a low protein diet *in utero*. As 5-HT is known as an essential growth and regulatory factor that drives the development and maturation of its own cellular network and related neuronal systems (Pino et al., 2004; Oberlander, 2012), an excess of 5-HT may lead to impaired interneuron migration and abnormal development of the serotonergic system itself and its target regions (Pino et al., 2004; Oberlander, 2012). Here we suggest that elevated hypothalamic levels of 5-HT in recuperated animals in early life cause developmental and functional alterations that increase susceptibility to obesity later in life.

We observed reduced maternal circulating tryptophan but increased 5-HT in pregnant dams fed a low-protein diet. This could be a consequence of (1) increased carbohydrate content in low-protein diet, added to balance protein reduction to obtain an isocaloric diet and (2) the dams' increased food intake in both absolute and relative terms (Fernandez-Twinn et al., 2003). Such dietary intervention can affect maternal insulin levels. Low-protein dams were shown to be hyperinsulinaemic at day 14 of pregnancy (Fernandez-Twinn et al., 2003). As circulating tryptophan levels decrease in an insulin-dose dependent manner, increased insulin concentration in these dams could contribute to the reduced tryptophan levels (Fernandez-Twinn et al., 2003; Fukagawa et al., 1987). Another possibility is that the dietary intervention has altered the gastrointestinal microbiome of the dam. Diet is the key regulator of both taxa and microbiota production of metabolites (Scott et al., 2012). More than 90% of 5-HT in the body is synthesized in the gut and microbiota has been shown to play a critical role in regulating host 5-HT biosynthesis and 5-HT blood levels (Yano et al., 2015).

Several mechanisms may be involved in mediating the relationship between reduced maternal circulating tryptophan and increased 5-HT in the brains of their unborn offspring. In adult rats food deprivation leads to an increase in the concentration of circulating free fraction of tryptophan (FFT), the fraction that passes through the blood-brain-barrier (Knott and Curzon., 1972; Perez-Cruet et al.,

1972). Raised circulating FFT and increases in tryptophan uptake by serotonergic neurons, activity of tryptophan-5-hydroxylase and synthesis of 5-HT have also been reported in intra-uterine growth restricted (IUGR) rat pups and infants (Kalyanasundaram, 1976; Hernandez et al., 1989; Manjerrez et al., 1996; Manjerrez et al., 1998). Up-regulation of FFT in IUGR was attributed to abnormal kinetics of tryptophan binding to albumin (Hernandez-Rodriguez et al., 2009). Reduced plasma levels of leucine, isoleucine and tyrosine which compete with tryptophan for entry into brain, may also contribute to an increase in transport of tryptophan across the blood-brain-barrier in IUGR pups (Roux and Jahchan, 1974).

In recent years, in both mice and humans, the placenta has emerged as an important source of 5-HT, that can directly impact on the fetal brain development (Bonnin et al., 2011). In our study the impact of maternal LP diet on 5-HT was more pronounced in the placenta and fetal brain than in the dam. This supports the proposal that, the placenta is cannibalized in response to maternal food deprivation to provide nutrients and substrates for the developing hypothalamus (Broad and Keverne, 2011). We observed an association between placental 5-HT and fetal brain 5-HT at E16.5, the time-point at which 5-HT fibres can be detected in the hypothalamus and both exogenous and endogenous sources of 5-HT contribute to fetal 5-HT synthesis (Aitken and Tork, 1988; Bonnin et al., 2011). Increased 5-HT production within the placenta may contribute to raised 5-HT levels in fetal brains from low protein fed dams. Maternal tryptophan levels have also been reported to influence central 5-HT levels in the offspring and maternal peripheral 5-HT proved to be important for offspring neurodevelopment (Bonnin and Levitt., 2011; Cote et al., 2007). This suggests that both maternal and placental tryptophan/5-HT metabolism may be an important molecular pathway for the fetal programming of the 5-HT system and brain development.

Changes in 5-HT bioavailability not only impact on hypothalamic circuitry formation during development but also have been shown to influence appetite and body weight in the adult (Porto et al., 2009; Berg et al., 2013; Madden and Zup 2014). 5-HT primarily regulates energy balance via activity at the G_q-coupled receptor, 5-HT_{2C}R. Here we report that elevated fetal 5-HT could potentially program decreased hypothalamic 5-HT_{2C}R expression, although direct evidence for this relationship remains to be established. The reduction in 5-HT_{2C}R may be an early and permanent developmental consequence of central hyperserotonemia. Reduced 5-HT_{2C}R function is known to promote increased appetite and obesity. Specifically, genetic inactivation of *Htr2c* that codes for 5-HT_{2C}Rs, leads to lifelong hyperphagia and elevated body weight in mice fed standard chow diet (Tecott et al, 1995), while pharmacological activation of these receptors reduces food intake and decreases body weight (Heisler et al., 2002; Halford and Harrold, 2012; Burke et al., 2014). 5-HT_{2C}Rs are involved in mediating the action of d-fenfluramine, which stimulates the release of 5-HT and inhibits the re-uptake of 5-HT into nerve terminals (Vickers et al., 2001; Trifunovic and Reilly, 2006; Xu et al., 2010). Moreover, the subpopulation of 5-HT_{2C}R expressed solely with POMC is sufficient to mediate D-fenfluramine and 5-HT_{2C}R agonist mCPP appetite suppression (Xu et al., 2008). Following

the administration of D-fenfluramine into the third ventricle we observed impaired sensitivity to 5-HT-stimulated food reduction in 3-month-old recuperated animals. As the functional capacity of 5-HT_{2C}R is dependent upon availability of active membrane receptor pools, the impaired response to D-fenfluramine in recuperated animals is likely a consequence of the reduced 5-HT_{2C}R protein expression in the hypothalamus of these animals.

We used LCM of the ARC combined with microarray analysis to identify differentially expressed genes that could be affected by early hyperserotonemia. The expression of KHSRP, which is involved in the control of mRNA decay (Gherzi et al., 2006; Ruggiero et al., 2007) and plays a key role in the translation of DNA damage signalling to miRNA biogenesis (Trabucchi et al., 2009; Zhang et al., 2011) was increased in 3-month-old recuperated offspring. Although recuperated animals have reduced longevity (Jennings et al., 1999), which is associated with increased oxidative stress and an impaired response to DNA damage (Tarry Adkins et al., 2008; Tarry Adkins et al., 2009) the functional significance of the increase in KHSRP in the brain of these animals remains to be determined.

5-HT_{2A}R is another gene that was increased in the 3-month-old recuperated offspring. The precise role that *5-HT_{2A}R* plays in food intake regulation has not been fully defined. *5-HT_{2A}R* was up-regulated in the ARC of diet-induced obese rats (Park et al., 1999), while in humans, 5-HT_{2A}R correlated positively with BMI and a polymorphism in the gene encoding 5-HT_{2A}R has been associated with obesity (Rosmond et al., 2002; Erritzoe et al., 2009; Carr et al., 2013). However, knockout of *5-HT_{2A}R* in mice does not alter food consumption or body weight gain when mice are fed laboratory chow (Weisstaub et al., 2006), suggesting the presence of developmental compensatory mechanisms or that body weight gain only occurs when these mice are placed on an obesogenic diet. There is little information regarding the precise neuronal location of 5-HT_{2A}R within the ARC. Here we report that 5-HT_{2A}Rs, like 5-HT_{2C}Rs (Heisler et al., 2002; Lam et al., 2008), are anatomically positioned to influence the activity of the critical energy balance regulator POMC. The extent to which the activity of POMC neurons can be influenced by action at 5-HT_{2A}Rs and whether this mechanism is altered in recuperated offspring as well as direct evidence for linking fetal hyperserotonemia to alteration of 5-HT_{2A}R in later life remain to be established.

As *5-HT_{2A}R* expression was increased in the ARC of recuperated pups at weaning and in adulthood but not in brains of fetuses or neonates, the differences in 5-HT_{2A}R must be established during the period of catch-up growth, between day 3 and day 22 of life, much later then the observed differences in 5-HT_{2C}R levels. Therefore, up-regulation of 5-HT_{2A}R may act as a secondary, counter-regulatory response to impaired 5-HT_{2C}R signalling. Counter-regulatory mechanisms have been reported among 15 existing 5-HT receptor subtypes as genetic alteration of one specific 5-HT receptor subtype can result in compensatory signalling via another, for example signalling through 5-HT_{1B}R in the absence of 5-HT_{2C}R (Heisler and Tecott, 2000; Dalton et al., 2004). It is possible that counter-regulatory up-regulation of 5-HT_{2A}R in response to impaired signalling via 5-HT_{2C}R only

emerges when growth-restricted newborn pups are born into and exposed postnatally to an environment of plentiful food. Some effects of early alterations in 5-HT therefore, may only appear in the presence of a particular postnatal environment. This has been shown to be true in a study of selective serotonin reuptake inhibitor-exposed 3-month-old infants, showing altered HPA stress response patterns which only became apparent when the method of infant feeding was taken into consideration (Oberlander et al., 2008).

5-HT_{2A}R stimulation via agonist administration produced a greater effect on appetite in recuperated rats, revealing that the over-expressed ARC 5-HT_{2A}Rs are functional. Since pharmacological stimulation of endogenous 5-HT was less effective in suppressing appetite in recuperated rats, these results suggest that the endogenous circuitry is insufficient to appropriately modulate 5-HT-regulated appetite. However, this endogenous programming change may be circumvented and the up-regulation of the 5-HT_{2A}R may be capitalized upon through pharmacological treatment with a 5-HT_{2A}R non-hallucinogenic agonist. The anorectic therapeutic profile could be potentially further improved by combining the 5-HT_{2A}R agonist, with 5-HT_{2C}R and 5-HT_{1B}R agonists as the co-application of 5-HT_{2C}R and 5-HT_{1B}R agonists produced a significant increase in the activity of POMC in the ARC (Doslikova et al., 2013). Development of a non-hallucinogenic 5-HT_{2A}R agonist may seem to be challenging however, studies showing that the glutamate mGlu2 receptor heterocomplex with 5-HT_{2A}R, and not 5-HT_{2A}R on its own, acts as a molecular target for the actions of hallucinogenic drugs provide a plausible avenue to explore in the development of therapeutically suitable5-HT_{2A}R agonist (Moreno et al., 2012).

Although we concentrated on studying mechanisms that potentially could mediate the effects of early elevated 5-HT levels on obesity risk in later life, early perturbations in the 5-HT neurotransmitter system are known to have major implications for mental health and behaviour in childhood and adulthood. Increased anxiety-like behaviour in nonhuman primate offspring of dams fed high fat diet was associated with perturbations in 5-HT system during fetal life (Sullivan et al., 2010). Early hyperserotonemia, in particular, has been identified as a potential factor in the pathogenesis of Autism Spectrum Disorder and Schizophrenia (Chugani, 2004; Madden and Zup, 2014). In addition, both 5-HT_{2A}R and 5-HT_{2C}R that are expressed throughout the brain have been implicated in the pathophysiology of psychiatric disorders (Abramowski et al., 1995; Hoyer et al., 1986; Heisler et al., 2007; Mestre et al., 2013; Lyddon et al., 2013]. 5-HT_{2C}Rs for example, was shown to play a role in addiction and reward behavior via modulating dopamine transmission within the mesolimbocortical dopaminergic system (Katsidoni et al., 2011). Dysregulation of the reward-related neurotransmitter systems and behaviours could also contribute to the increased susceptibility to obesity in the recuperated offspring (Grissom et al., 2014)

In summary, we report that elevated 5-HT in fetuses exposed to maternal protein restriction, may underlie a permanent reduction in 5-HT_{2C}R expression and function and secondary counter-regulatory up-regulation of 5-HT_{2A}R and increased sensitivity to 5-HT_{2A}R agonist. Thus, our

results not only identify a molecular mechanism through which maternal diet may impair offspring energy balance but also point to a promising pharmacological strategy with 5-HT_{2A}R agonist medication to correct this impairment.

Materials and Methods

Experimental groups and tissue collection

All procedures involving animals were conducted in accordance with the University of Cambridge and the University of Buckingham project licences under the UK Home Office Animals (Scientific Procedures) Act (1986). The breeding of animals was conducted at both the University of Cambridge and the University of Buckingham. Five cohorts of Wistar rats (*Rattus norvegicus*) were established for the studies: 1st for E16.5 experimental measures, 2nd for laser-capture microdissection (LCM) and microarray validations, 3rd for *in situ* hybridization, 4th for intracerebroventricular (i3v) and 5-HT/tryptophan measurements and 5th for protein and mRNA analysis. Detailed information regarding the diet composition and the set-up of the maternal protein restricted and control dams have been published previously (Cripps et al., 2009; Berends et al., 2013). Briefly, on postnatal day 3 (P3) two experimental groups of offspring were established: controls [offspring of control dams (20% protein, w/v), culled to eight (four males and four females) suckled by control dams] and recuperated [offspring of dams fed a low-protein diet (8% protein, w/v) during pregnancy, but nursed by control dams, culled to four males to maximize the plane of nutrition]. The animals were allocated to experimental groups at random. Whole heads were collected from fetuses at E16.5 of pregnancy and from male pups at P3. In addition hypothalami were dissected from brains of male offspring at P3. The body weight of the remaining pups was recorded at P7, P14 and P21. Following weaning at day 22, one male per litter was culled by a rising concentration of CO_2 and the brain was dissected. After weaning, the remaining males were fed standard laboratory chow and body weight and food intake were recorded weekly. At 3 months of age males were culled and brains were collected. All the dissected brains and heads were frozen on powdered dry ice and were stored at -80°C until further processing. Amniotic fluid and placentas were harvested at E16.5 and serum was prepared from the dams and male pups at P3, weaning and 3 months of age, and stored at - 80°C prior to the measurement of 5HT and tryptophan levels.

5-HT and tryptophan assay

Whole brains, placentas, amniotic fluid and serum were used for measurement of 5-HT (DRG International, Inc, New Jersey, USA) and tryptophan (Abnova, Heidelberg, Germany) by ELISA following the manufacturer's recommendation for sample preparation, acylation (5-HT) and derivatisation (tryptophan). For the measurement of 5-HT in tissue, hypothalamic blocks were excised from frozen brains and extracted as described (Huang et al., 2012). Briefly, samples were homogenised and deproteinised for 30 min in 0.2 N perchloric acid solution containing 7.9 mM $Na_2S_2O_5$ and 1.3 mM disodium ethyleneamine-tetra-acetic acid. The homogenate was centrifuged at 10,000 g for 10 min at 4 ^{0}C and the supernatant assayed for 5-HT.

Feeding studies

Animals were cannulated as described previously (Stocker et al., 2012). Briefly a cannula was inserted into the third ventricle under a gaseous anaesthetic (isofluorane: Isoba, Schering-Plough Animal Health) using coordinates from the stereotactic rat brain atlas (Paxinos and Watson, 1998). Its position was verified by a positive drinking response over 15 min to angiotensin II (20 μgml^{-1} in 2.5 μl). For measurements of acute effects of D-fenfluramine or 5-HT$_{2A}$R agonism on food intake, 3-month-old rats were individually housed, fasted for 4 h, dosed at the beginning of the dark period and re-fed. Peptide (250 nmoles) was given in 2.5 μl saline. Animals were dosed using a Latin square design. 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 the D-fenfluramine (Tocris Bioscience, Bristol, UK), or the high affinity 5-HT$_{2A}$R receptor agonist TCB-2 (Tocris Biosciences, Bristol, UK) were based on published data (Vickers et al., 1999; Vickers et al., 2001; Trifunovic and Reilly, 2006; McLean et al., 2006; Xu et al., 2010, Fox et al., 2010) and doses were optimised by ourselves.

Western blotting

Hypothalami were dissected from frozen brains according to landmarks: anterior to the optic chiasma; posterior to the mammillary bodies; lateral at the hypothalamic sulcus; and superior to the anterior commissure. Dissected hypothalami from day-3 and 3-month-old male offspring were homogenised in TK lysis buffer and Western blotting analysis was carried out as previously described (Martin-Gronert et al., 2008). Anti-goat primary antibody against 5-HT$_{2C}$R was purchased from Santa Cruz Biotechnology (cat sc-15081, lot D1114, Santa Cruz, USA) and 1:200 dilution was used. The antibody was validated in two previous studies (Bubar et al., 2005; Anastasio et al., 2010). Horseradish peroxidase-conjugated secondary antibody [anti-goat antibody (Jackson ImmunoResearch, Stratech, Newmarket, UK)] was used at 1:10000 dilution.

Laser-capture microdissection (LCM), RNA isolation and analysis

Hypothalamic sections of the ARC were prepared on a cryostat at 14μm thickness from approximately -4.52 to -2.30 mm relative to Bregma (Paxinos and Watson, 1998). Sections were collected onto RNase-free membrane-coated slides (P.A.L.M) that had been baked at 200^{o}C for 4 hrs and UV cross-linked for 30 min. Within 24 hours of sectioning, sections were placed for 30 s each time in 95%, 75% and 50% ethanol for rehydration. Sections were stained with 1% cresyl violet stain (Ambion) for 1 min, dehydrated in graded ethanol concentrations (50%, 75% and twice in 100% for 30 s each time), placed in HistoClear (National Diagnostics) for 5 min and air dried. LCM was performed using a P.A.L.M. MicrolaserSystem (P.A.L.M. Microlaser Technologies) (Figure 4A). 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 Agilent BioAnalyzer PicoChips (Agilent Technologies Inc, Santa Clara, CA, USA). Total RNA was isolated from E16.5 fetal heads and neonatal and adult (3 months of age) hypothalami as previously described (Zaibi et al., 2010) and analyzed using a NanoDrop ND1000 (Thermo Fisher Scientific, Delaware, USA).

RNA amplification

An ovation Pico RNA Amplification System (Nugen Technologies Inc, San Carlos, CA, USA) was used for the amplification of RNA destined for microarray analysis. RNA amplification of LCM ARC samples 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). 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, San Carlos, CA, USA) using the Affymetrix GeneChip protocol to fragment and label the target, ready for hybridization to the arrays (Affymetrix 2004). 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 the University of Warwick, using n=6 chips per group.

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. All downstream analysis of microarray data was performed using GeneSpring GX 12.0 (Agilent). The *CEL* files were used for the RMA (Irizzarry et al., 2003) and GC-RMA (Wu et al., 2004) analyses, while the pivot files were used for GCOS analysis. After importing the data, each chip was normalized to the 50th 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 down-regulated if the 1.3-fold threshold was reached and $P<0.05$. Only genes that met the above criteria using three different algorithms: 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. Functional analysis was performed using Ingenuity Pathway Analysis (Ingenuity Systems Inc).

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. The data was normalized to *Ppia (cyclophilin)* expression which did not change between maternal treatment groups. Real time PCR (StepOne^{TM}, Applied Biosystems, Paisley, UK) was carried out using Assay-on-Demand pre-designed primer and probe sets (Applied Biosystems, Paisley, UK). Data were analyzed using the comparative ΔC_t method, comparing recuperated animals with controls. All procedures were carried out in accordance to the manufacturer's recommendation.

In Situ Hybridization Histochemistry (ISHH)

Coronal sections (20 μm) of frozen hypothalamic ARC, obtained using a cryostat, were thaw-mounted onto poly-L-lysine slides (Polysine, Menzel Glaser, Braunschweig, Germany). Ten sets of slides per animal were serially collected, with the first set of 10 beginning at approximately -4.52 and ending at -2.12 mm, relative to Bregma, according to the atlas of the rat brain (Paxinos and Watson). For the sectioning of neonatal rat brains whole heads were used and the sectioning was guided by the neonatal rat brain atlas (Ramachandra and Subramanian, 2011). Slides were stored at –80 °C until use.

A [^{35}S]-radiolabelled riboprobe targeting nucleotides 1700-1910 of the rat 5-HT_{2A}R mRNA transcript was generated by PCR from whole rat brain cDNA. The 210 bp fragment was cloned into pCR-TOPO4 (Life Technologies, Paisley, UK). For antisense probe generation, Pst1 linearised recombinant plasmid was subjected to *in vitro* transcription using T7 polymerase in the presence of ^{35}S-labelled UTP, as per manufacturer's instructions (Ambion). The ISHH procedure used has been described in detail previously (Alon et al., 2009; Garfield et al., 2012). Autoradiographic images were quantified using Image Proplus software (Media Cybernetics, MD 20910). Standard curves were generated from ^{14}C autoradiographic microscales (Amersham, UK) and integrated optical density (IOD) and area of the hybridization signal were measured. For each animal, 3-5 sections of ARC (-4.52mm to -2.12 mm from bregma) and 2-3 sections for VMN (-3.60mm to -2.12mm from bregma) were analyzed. Average ARC and VMH *5-HT_{2A}R* mRNA expression was calculated.

Dual-label ISHH and immunohistochemistry (IHC)

To assess co-localisation of 5-HT_{2A}R and POMC, brains taken from control rats, were first processed for detection of *5-HT_{2A}R* mRNA by ISHH, as described above. Following this, the tissue was washed in PBS before commencement of the IHC protocol to label α-melanocyte-stimulating hormone (MSH) protein using procedures previously described (Alon et al., 2009; Garfield et al., 2012). Briefly, sections were incubated in 0.3% H_2O_2 in PBS, then rinsed in PBS, blocked in 0.5% BSA/ 0.5% Triton-X 100 in PBS and left in blocking buffer containing rabbit α-MSH antibody (1/10,000; Chemicon, Millipore, MA, USA) overnight. Tissue was then washed in PBS and a biotinylated donkey anti-rabbit secondary antibody (Vector Laboratories, CA, USA) was applied at 1/1000 in blocking buffer. Sections were then washed in PBS, incubated in VectaStain ABC reagent, and following this, chromogenic detection was conducted using 3,3'-diaminobenzidine (DAB) reagent (Vector Laboratories). Sections were mounted onto superfrost slides, dried, then dipped in photographic emulsion (Kodak) and stored at 4°C for two weeks before being developed using Kodak developer and fixer. Double labelled cells were recorded if α-MSH-immunoreactive (IR) positive cell bodies were overlaid with a ^{35}S *5-HT_{2A}R* signal greater than 3X background.

Statistical analysis

Two-tailed unpaired Student's *t*-test was used for statistical analysis and the data are presented as mean±s.e.m. unless otherwise stated. Fractional growth rates were calculated using formula: fractional growth rate = (current - starting weight)/(period x starting weight). For microarray data *p*-values were calculated using a two-tailed *t*-test. All data were analyzed using GraphPad Prism (Graphpad, La Jolla, USA). Number (n) refers to number of litters used. $P<0.05$ was considered statistically significant.

Data availability

GSE76012 study at:

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76012

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.S.M-G., C.J.S., E.T.W., R.L.C, M.A.C., J.R.S.A., G.S.H.Y., L.K.H. and S.E.O. contributed to the conception, design, and interpretation of the data. M.S.M-G., C.J.S., E.T.W., R.L.C., A.S.G., Z.J., G.D. and G.S.H.Y. helped acquire the data. M.S.M-G., C.J.S., E.T.W., Z.J. analysed the data. M.S.M-G., L.K.H. and S.E.O. wrote the manuscript. All authors have revised the final version of the manuscript.

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Figures

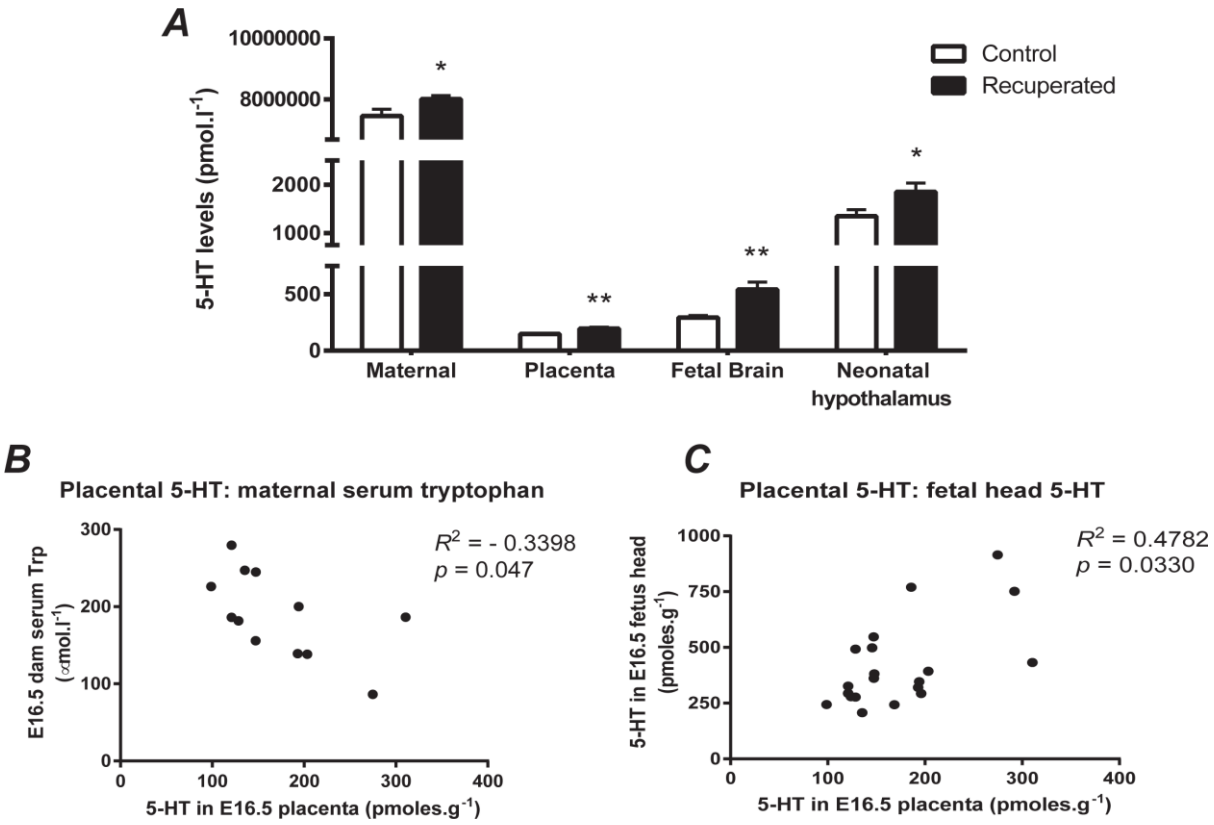

Figure 1. Effects of maternal protein restriction on 5-HT levels in pregnant rat dams. (A) 5-HT levels in maternal serum, the placenta, fetal brain (all analyzed at E16.5) and neonatal hypothalamus. Data analyzed using unpaired Student's t test and presented as % mean control, for maternal serum 5-HT n=6, placenta 5-HT n=16, fetal brain 5-HT n=10, neonatal hypothalamus n=12 per group. (**B, C)** Correlations between maternal, placental and fetal 5-HT levels. (**B**) Correlation between placental 5-HT and maternal serum tryptophan, analyzed using Pearson correlation coefficient as data was normally distributed, n=6 per group. (**C)** Correlation between placental 5-HT and fetal head 5-HT analyzed using Spearman correlation coefficient as data was not normally distributed. n=10 per group, n represents number of litters. * $P<0.05$; ** $P<0.01$.

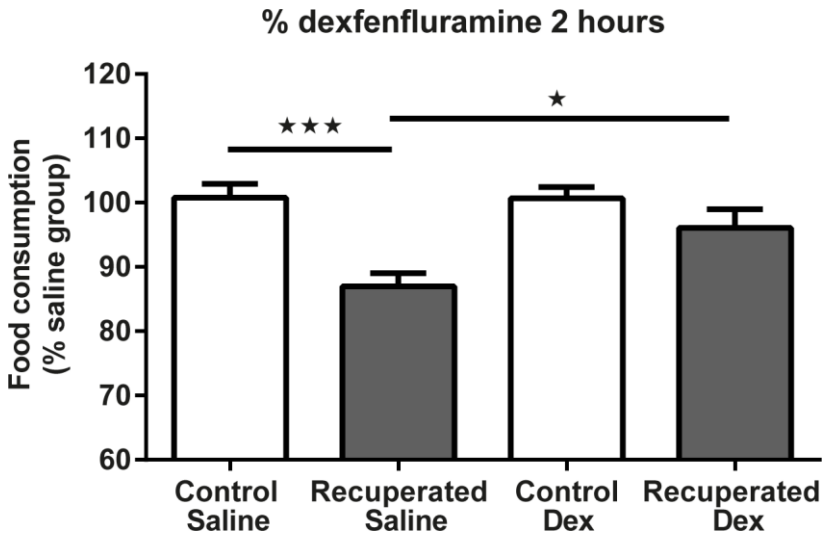

Figure 2. Effects of maternal protein restriction on food intake in 3-month-old offspring following central administration of D-fenfluramine (Dex). Food consumption during the 2 hours following lights out expressed as a percentage of the saline dosed group. Difference between the groups analyzed by one-way ANOVA to saline for control and recuperated offspring. n=6 per group with n representing number of litters. The following numbers of animals were used for each experimental group: control saline =18 rats, control Dex =20, Recuperated saline =17 and Recuperated Dex =20 rats. D-fenfluramine was administered at the dose of 250 nmoles in 2.5 ul of saline. * P<0.05 for Control Dex versus Recuperated Dex. *** P<0.001 for Control saline versus Control Dex.

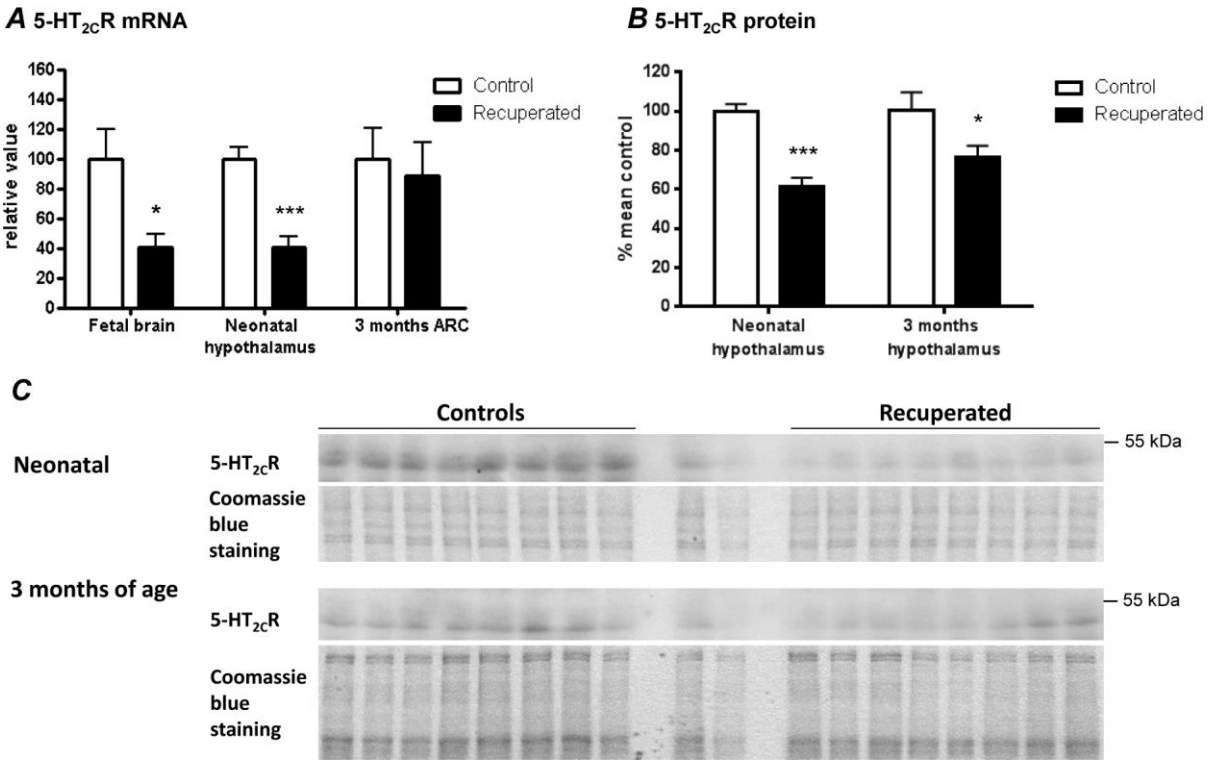

Figure 3. Effects of maternal protein restriction on central 5-HT$_{2C}$R mRNA and protein expression in the offspring. (**A)** 5-HT$_{2C}$R mRNA levels as measured using qRT-PCR. Gene expression data was normalized to *Ppia.* (**B)** 5-HT$_{2C}$R protein levels analyzed using Western blotting. **(C)** Western blots. 20 mg and 10 mg of pooled samples were loaded in the middle of the gels to ensure the linearity of the signal. Data analyzed using unpaired Student's t test and presented as % mean control±s.e.m. n=8 per group, n represents number of litters * P<0.05; *** P<0.001.

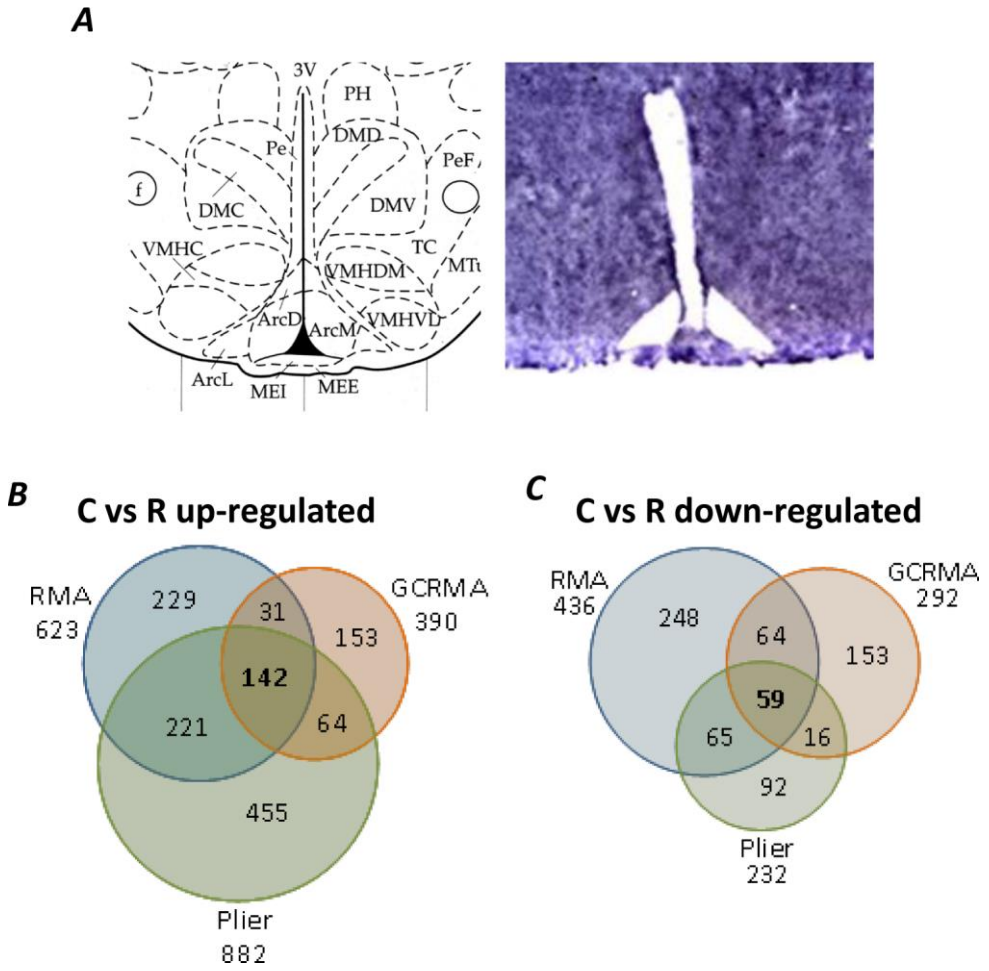

Figure 4. Laser-capture microdissection (LCM) of the hypothalamic ARC of 3-month-old rats and microarray analysis results. (**A**) LCM of the ARC (-4.52 to – 2.30 mm relative to Bregma). Brain Atlas image was taken from Paxinos and Watson (1998). **(B, C**) Venn diagrams showing the effects of maternal protein restriction followed by catch up growth on the expression of genes in the ARC of male 3-month-old offspring, according to three different, robust analyses: GeneChip Operating Software (GCOS), GeneChip Robust Multi-array Averaging (GC-RMA) and Robust Multi-array Averaging (RMA). Genes were considered to be up- or down-regulated if the 1.3-fold threshold was reached and $P<0.05$. **(B)** Up-regulated genes and **(C)** Down-regulated genes in the recuperated animals when compared to controls. The sizes of circles and numbers in parentheses indicate the number of genes as identified by either the GCOS, RMA or GC-RMA algorithms. For microarray analysis n=6 chips per group, n represents number of litters.

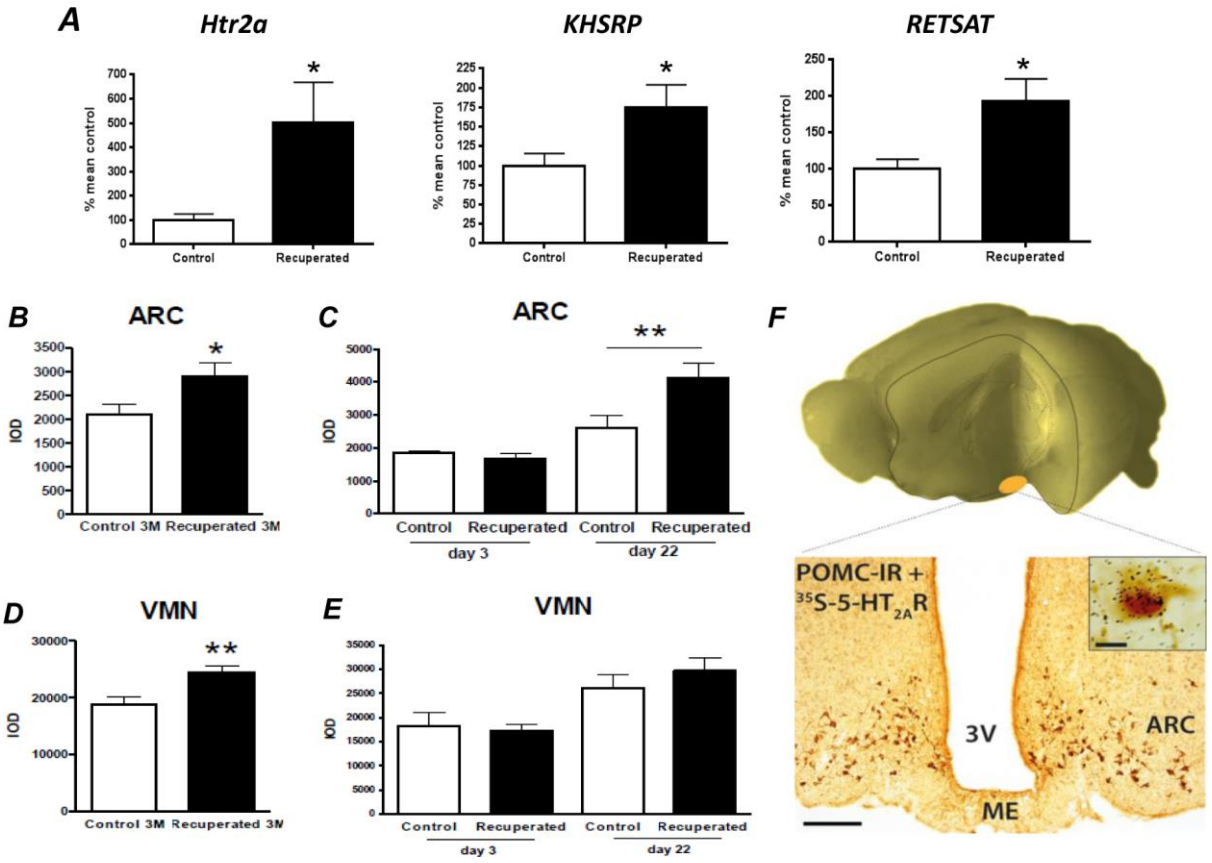

Figure 5. Validation of the differentially expressed genes identified using microarray approach as being regulated by early nutrition. (**A)** Validation of the differentially expressed genes in the ARC of 3-month-old control and recuperated offspring. Analysis carried out using Taqman qRT-PCR. Gene expression data was normalized to *Ppia.* Data analyzed using a two-tailed unpaired Student's t-test. (**B**) Validation of 5-HT_{2A}R mRNA expression in the ARC using ISHH. (**C)** 5-HT_{2A}R mRNA expression in rats at 3 and 22 days of age in the ARC. **(D)** 5-HT_{2A}R mRNA expression in 3-month-old rats in the VMN using ISHH. (**E)** 5-HT_{2A}R mRNA expression in 3 and 22 day old rats in the VMN. (**F)** Localisation of 5-HT_{2A}R on ARC POMC neurons in 3-month-old Control rats as detected using ISHH for ^{35}S-5-HT_{2A}R and chromogenic IHC for α-MSH. Dense black granular staining directly overlapping brown α-MSH cell body and axons indicates the presence of 5-HT_{2A}R on POMC neurons. Scale bar for ARC image represents 200 μm, in the inset 10 μm. for **(B, D)** Data were analyzed using two-tailed Student t-test, for **(C, E)** A two-way ANOVA was used with appropriate Duncan's posthoc test. Values are expressed as means±s.e.m. For **(A, B, D)** n=8 per group, for **(C, E)** n=6-8 per group, n represents number of litters. * P<0.05; ** P<0.01.

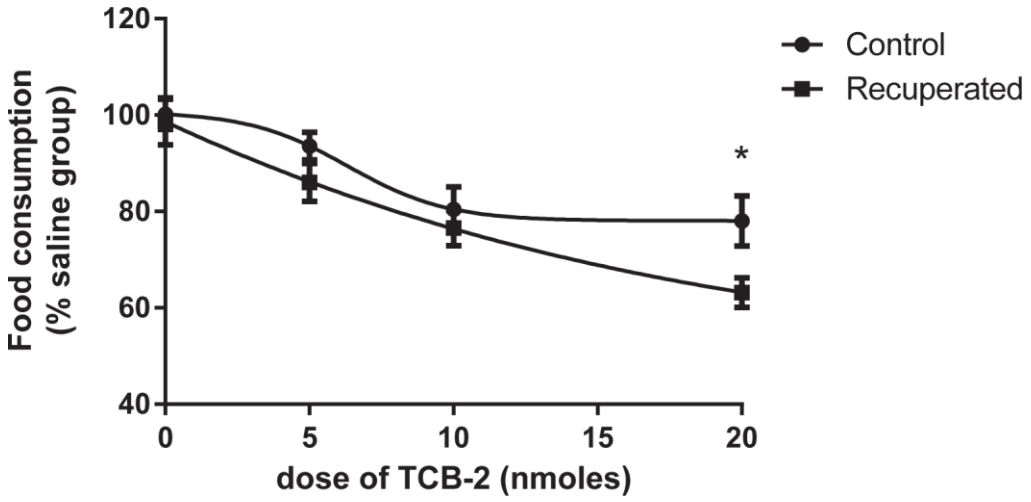

Figure 6. Effects of maternal protein restriction on food intake following central administration of the 5-HT_{2A}R agonist (TCB2) in 3-month-old rats. Food consumption during the 2 hours following lights out expressed as a percentage of the saline dosed group. Differences between the groups were analyzed using two-way ANOVA (overall effect of TCB2 administration, $P<0.001$; overall effect of early nutrition, $P<0.05$) followed by Bonferroni's multiple comparison test. n= 13-15 per group, n represents number of litters. * $P<0.05$

Table 1. Growth trajectories, body weights and brain weights

A. Growth trajectory during postnatal life

Body weight (g)	Control	Recuperated
P3	7.4 ± 0.3	6.2 ± 0.2***
P7	15.9 ± 0.8	13.4 ± 1.1*
P14	32.0 ± 1.1	32.5 ± 1.4
P21	52.2 ± 1.4	55.5 ± 1.4

B. Body and brain weights after weaning

	Control	Recuperated
Body weight (g) at 3M	417 ± 9.0	417 ± 7.9
Brain weight (g) at P22	1.33 ± 0.11	1.42 ± 0.02
Brain weight (% of BW) at P22	2.90 ± 0.08	2.77 ± 0.08
Brain weight (g) at 3M	2.00 ± 0.03	1.97 ± 0.05
Brain weight (% of BW) at 3M	0.48 ± 0.01	0.47 ± 0.01

Data analyzed using Student's t test. Values are expressed as means±s.e.m. n =10 per group, * P<0.05; *** <0.001

Table 2. Genes in the ARC that are differentially expressed between the recuperated and control 3-month-old male rats

(A) Top 25 genes increased in Recuperated offspring compared to Controls

Probe Set ID	Fold Change	Gene Symbol	Gene Title
1387752_at	**2.55**	**Htr2a**	5-hydroxytryptamine (serotonin) receptor 2A
1379905_at	**1.91**	**Gtpbp1**	GTP binding protein 1
1377472_at	**1.78**	**Extl1**	exostoses (multiple)-like 1
1389755_at	**1.71**	**Cdca7l**	cell division cycle associated 7 like
1376525_at	**1.69**	**Khsrp**	KH-type splicing regulatory protein
1393134_at	**1.69**	**Sephs1**	selenophosphate synthetase 1
1394434_at	**1.68**	**Snf1lk2**	SNF1-like kinase 2
1383843_at	**1.66**	**Hlcs**	holocarboxylase synthetase
1377726_at	**1.59**	**Trim25**	tripartite motif-containing 25
1381410_at	**1.58**	**Fgd5**	FYVE, RhoGEF and PH domain containing 5
1383606_at	**1.56**	**Tc2n**	tandem C2 domains, nuclear
1379817_at	**1.53**	**Purg**	purine-rich element binding protein G
1371356_at	**1.51**	**Tenc1**	tensin like C1 domain containing phosphatase (tensin 2)
1370806_at	**1.51**	**Retsat**	retinol saturase (all trans retinol 13,14 reductase)
1378776_at	**1.50**	**Pou6f1**	POU class 6 homeobox 1
1395261_at	**1.49**	**Snrp70**	U1 small nuclear ribonucleoprotein polypeptide A
1369601_at	**1.49**	**Nyw1**	ischemia related factor NYW-1
1381919_at	**1.48**	**Hps6**	Hermansky-Pudlak syndrome 6
1396053_at	**1.47**	**Nedd9**	neural precursor cell expressed
1378282_at	**1.47**	**Csnk2a2**	casein kinase 2, alpha prime polypeptide
1389632_at	**1.46**	**Rhobtb1**	Rho-related BTB domain containing 1
1368561_at	**1.45**	**Abcd2**	ATP-binding cassette, sub-family D (ALD), member 2
1393320_at	**1.45**	**Utp15**	UTP15, U3 small nucleolar ribonucleoprotein
1390506_at	**1.44**	**Med1**	mediator complex subunit 1
1376423_at	**1.44**	**Fbxl19**	F-box and leucine-rich repeat protein 19
1376565_at	**1.33**	**Pde4a**	Phosphodiesterase 4A

(B) Top 25 genes decreased in Recuperated offspring compared to Controls

Probe Set ID	Fold Change	Gene Symbol	Gene Title
1387675_at	**2.48**	**Plau**	plasminogen activator, urokinase
1382284_at	**2.09**	**Nek3**	NIMA (never in mitosis gene a)-related kinase 3
1384767_at	**1.80**	**Usp42**	ubiquitin specific peptidase 42
1375362_at	**1.74**	**Sppl2a**	signal peptide peptidase-like 2A
1372995_at	**1.70**	**Prkd2**	protein kinase D2
1398431_at	**1.67**	**Car8**	carbonic anhydrase 8
1371770_at	**1.63**	**RT1-CE4A2**	prefoldin 6
1369638_at	**1.62**	**Eef2k**	eukaryotic elongation factor-2 kinase
1370204_at	**1.59**	**Frag1**	FGF receptor activating protein 1
1396268_at	**1.58**	**Sp110**	SP110 nuclear body protein
1391669_at	**1.57**	**Ptprb**	protein tyrosine phosphatase, receptor type, B
1385871_at	**1.54**	**Dhx36**	DEAH (Asp-Glu-Ala-His) box polypeptide 36
1388030_at	**1.48**	**RT1-M6-1**	gamma-aminobutyric acid (GABA) B receptor 1
1392174_at	**1.47**	**Chst12**	carbohydrate sulfotransferase 12
1376644_at	**1.46**	**Med19**	mediator complex subunit 19
1388705_at	**1.44**	**RGD1565037**	selenoprotein M
1389123_at	**1.44**	**Ccl6**	chemokine (C-C motif) ligand 6
1370384_at	**1.43**	**Prlr**	prolactin receptor
1372270_at	**1.43**	**Dpagt1**	GlcNAc-1-P transferase
1373109_at	**1.43**	**Map2k7**	mitogen activated protein kinase kinase 7
1375137_at	**1.38**	**Arpc2**	actin related protein 2/3 complex, subunit 2
1393172_at	**1.35**	**Nab1**	Ngfi-A binding protein 1
1388088_at	**1.34**	**Usf2**	upstream transcription factor 2
1397642_at	**1.34**	**Rad50**	RAD50 homolog (S. cerevisiae)
1398242_at	**1.34**	**Ppp5c**	Protein phosphatase 5, catalytic subunit

(C) RMA Plier gene function analysis for Recuperated offspring compared to Controls

Category	p value
Cell Cycle	7.01E-05
Connective Tissue Development and Function	7.01E-05
Cellular Growth and Proliferation	1.64E-04
Drug Metabolism	1.64E-04
Molecular Transport	1.64E-04
Small Molecule Biochemistry	1.64E-04
Cancer	1.65E-04
Neurological Disease	2.34E-04
Cellular Development	3.55E-04
Cellular Assembly and Organization	4.88E-04

Fold-changes were calculated by comparison of the mean expression value in the 3-month old recuperated offspring to the age-matched control group. $P<0.05$ for all the genes identified.