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## Short Communication

# Ghrelin and hypothalamic NPY/AgRP expression in mice are affected by chronic early-life stress exposure in a sex-specific manner



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## ABSTRACT

Early-life stress (ES) is a risk factor for metabolic disorders (e.g. obesity) with a notoriously higher prevalence in women compared to men. However, mechanisms underlying these effects remain elusive. The development of the hypothalamic feeding and metabolic regulatory circuits occurs mostly in the early sensitive postnatal phase in rodents and is tightly regulated by the metabolic hormones leptin and ghrelin. We have previously demonstrated that chronic ES reduces circulating leptin and alters adipose tissue metabolism early and later in life similarly in both sexes. However, it is unknown whether chronic ES might also affect developmental ghrelin and insulin levels, and if it induces changes in hypothalamic feeding circuits, possibly in a sex-dependent manner. We here show that chronic ES, in the form of exposure to limited nesting and bedding material from postnatal day (P)2 to P9 in mice, affects ghrelin levels differently, depending on the form of ghrelin (acylated vs desacylated), on age (P9 vs P14) and on sex, while insulin levels were similarly increased in both sexes after ES at P9. Even though ghrelin levels were more strongly affected in ES-exposed females, hypothalamic neuropeptide Y (NPY) and agouti-related peptide (AgRP) fiber density at P14 were similarly altered in both sexes by ES. In the paraventricular nucleus of the hypothalamus, both NPY and AgRP fiber density were increased, while in the arcuate nucleus of the hypothalamus, NPY was increased and AgRP unaltered. Additionally, the hypothalamic mRNA expression of ghrelin's receptor (i.e. growth hormone secretagogue receptor) was not affected by ES. Taken together, the specific alterations found in these important regulatory circuits after ES might contribute to an altered energy balance and feeding behavior in adulthood and thereby to an increased vulnerability to develop metabolic disorders.

## 1. Introduction

The incidence of obesity has risen dramatically over the last few decades. A growing body of evidence suggests that the perinatal environment greatly modulates the risk of such disorders. In particular, (pre-)clinical studies suggest that not only adverse early-life nutrition, but also exposure to other forms of early-life stress (ES), enhance the risk of metabolic disorders in adulthood (Murphy and Loria, 2017; Yam et al., 2017). Yet, the exact mechanisms leading to such vulnerability are unknown.

Metabolic processes are largely regulated by the arcuate nucleus of the hypothalamus (ARH) (Bouret and Simerly, 2006). Neurons in the ARH co-express the orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) and project to, among others, the paraventricular nucleus of the hypothalamus (PVH) to exert their metabolic effects. The development of these hypothalamic circuits, occurring mainly during

the first two postnatal weeks in rodents, is tightly controlled by leptin originating from the adipose tissue and possibly from ingested maternal milk during this early postnatal period (Bouret and Simerly, 2006; Nozhenko et al., 2015; Watez et al., 2017).

A leptin surge from approximately postnatal day (P)4 to P16 in rodents controls the formation of hypothalamic feeding and metabolic circuits, and disruptions of this surge alter ARH and PVH connections, which can result in long-term changes in body weight and adiposity (Yura et al., 2005). We have previously shown that chronic ES reduces circulating leptin at P9 (Yam et al., 2017), suggesting that ES might alter the hypothalamic circuits and thereby possibly contribute to the susceptibility to metabolic disorders.

There is emerging evidence that, next to leptin, ghrelin (mainly secreted by the stomach and possibly maternal milk), might also be involved in regulating the neurodevelopment of hypothalamic circuits (Nozhenko et al., 2015; Slupecka et al., 2016; Steculorum and Bouret,

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2011). Ghrelin increases gradually from P0 and reaches adult-like levels at P14 in rodents, and exists in the circulation as acylated ghrelin (AG) and desacylated ghrelin (DAG). To date, AG has received the most attention and is the natural ligand of the growth hormone secretagogue receptor (GHSR), which is highly expressed in ARH–NPY/AgRP neurons (Edwards and Abizaid, 2017). There is contradictory evidence for the exact role of AG in modulating the formation of the hypothalamic circuit, as AG has been shown to inhibit (Steculorum et al., 2015), but also to stimulate (Collden et al., 2015; Steculorum and Bouret, 2011) hypothalamic NPY/AgRP expression. As for DAG, its role has been neglected for a long time. However, there is emerging evidence supporting a biological function of DAG (Delhanty et al., 2012; Fernandez et al., 2016; Ku et al., 2016). Indeed, DAG injections increase the hypothalamic cFos expression (Asakawa et al., 2005; Fernandez et al., 2016) and has been shown to act on a subset of ARH neurons in adult mice, independently from GHSR (Fernandez et al., 2016). In addition, DAG impairs the orexigenic effects of peripherally administered ghrelin, indicating the important functional effects of DAG on the hypothalamus.

Very little is known about the effects of ES on the two forms of ghrelin and on the development of hypothalamic feeding circuits (Schmidt et al., 2006). Similarly, the effects of ES on insulin has so far only been described in rats during adulthood (Maniam et al., 2015a, b; Mela et al., 2012). Importantly, both ES and obesity show sex-dependent vulnerabilities (Murphy and Loria, 2017). However, studies addressing sex-specific effects of ES on hypothalamic feeding circuits and metabolism are rare and mostly focus on other aspects of metabolic regulation. We here therefore study the effects of chronic ES on total ghrelin, AG and DAG, insulin as well as hypothalamic NPY, AgRP protein and *GHSR* mRNA expression in young postnatal mice of both sexes. Understanding the sex-specific effects of ES on these systems will provide new insights into how ES might lead to the prominent sex-specific vulnerability to metabolic disorders.

## 2. Material and methods

Chronic ES (limited nesting and bedding material, P2–P9) was induced in C57Bl/6J mice as described earlier (Naninck et al., 2015; Rice et al., 2008; Yam et al., 2017). Briefly, litters were culled to six pups per dam (sex ratio of 3:3 or 4:2) without cross fostering and randomly allocated to a control (CTL) or stress condition till P9. Male and female mice were either sacrificed at P9, or transferred to standard cages and sacrificed at P14. All experiments were carried out in accordance with the Dutch legislation and European Union directives on animal experiments. Mice from 2 to 3 litters were included in each group at P9 (males: CTL = 3, ES = 7; females: CTL = 4, ES = 7) and P14 (males: CTL = 10, ES = 8; females: CTL = 5, ES = 6). Total ghrelin and AG were determined in treated serum by ghrelin ELISAs (Millipore, Billerica, MA, USA), and DAG levels estimated by subtracting AG from total ghrelin levels (Sominsky et al., 2017).

From this same P14 cohort, hypothalamic tissue was dissected and treated with TRIzol Reagent (Invitrogen, Breda, the Netherlands) to isolate RNA. We used 250 ng tissue to synthesize cDNA and determined hypothalamic GHSR expression (F:5'-GCTGCTCACCGTGATGGTAT-3'; R:5'-ACCACAGCAAGCATCTTCACT-3') by qPCR. All primer pairs were tested for PCR amplification efficiency and housekeeping gene stability was verified (gene stability value < 0.5; coefficients of variation < 0.2) using Biogazelle qBASE+ 3.0 software (Gent, Belgium). All data were normalized against the housekeeping genes  $\alpha$ -TUB and RPL13A (for details, see Yam et al., 2017).

From our previous P9 cohort where we measured leptin levels in Yam et al., 2017 (males: CTL = 13, ES = 12; females: CTL = 3, ES = 12), blood was collected to measure insulin levels (Milliplex Mouse Adipokine Multiplex, Millipore, Amsterdam, The Netherlands). A second P14 cohort was perfusion-fixed with 4% paraformaldehyde (males: CTL = 4, ES = 8; females: CTL = 4, ES = 5). Brains were

sliced into five series (40  $\mu$ m thick) and parallel series were immunostained for NPY (rabbit- $\alpha$ -NPY 1:500/24 h/4 °C, Sigma-Aldrich; goat- $\alpha$ -rabbit-488 1:500/2 h/RT) and AgRP (goat- $\alpha$ -AgRP 1:500/48 h/RT, Neuromics; rabbit- $\alpha$ -goat-594 1:200/12 h/RT). NPY and AgRP fiber density was determined within a defined region of interest using a thresholding method as described previously by Sominsky et al., 2017. Subsequently, the total fluorescence signal intensity was measured throughout the Z-stacks acquired by an upright confocal microscope (Nikon D-eclipse 90i-C1; NIS-elements software), and quantified in an average of 3–4 hypothalamic sections containing the ARH (from bregma: –1.46 and –2.18 mm) and PVH (from bregma: –0.94 and –1.22 mm).

Statistical analyses were carried out using SPSS 22.2 (IBM Software), independent *t*-test (for body weight of dams), two-way ANOVA (condition\*sex; for body weight offspring, insulin, NPY, AgRP and GHSR) and three-way ANOVA (condition\*sex\*age; for ghrelin levels), followed by a non-parametric Games-Howell *post-hoc* test, considering unequal variances and group sizes.

## 3. Results

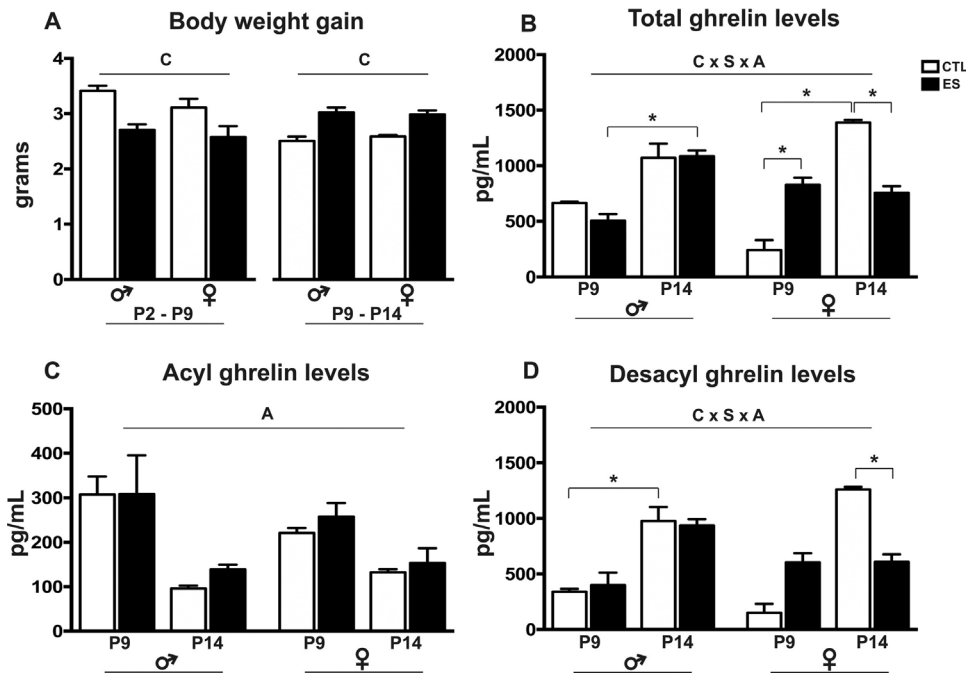
Chronic ES exposure resulted in no differences in the body weight gain from P2 to P9 in dams ( $t_{(7)} = -0.33$ ,  $p = 0.75$ ; data not shown), but reduced the P2 to P9 body weight gain in the offspring of both sexes (condition:  $F_{1,56} = 20.89$ ,  $p < 0.01$ ; Fig. 1a). In the period between P9 and P14, ES-exposed males and females demonstrated a higher body weight gain (condition:  $F_{1,27} = 36.12$ ,  $p < 0.01$ ; Fig. 1a), showing no differences in absolute body weight when compared to controls at P14.

These findings were accompanied by changes in ghrelin levels after ES, particularly in females. Total ghrelin level was increased at P14 when compared to P9 in CTL and ES-exposed males (age:  $F_{1,42} = 57.84$ ,  $p < 0.01$ ), and CTL females (condition\*age:  $F_{1,42} = 14.82$ ,  $p < 0.01$ , *post-hoc*:  $p < 0.01$ ), but not in ES females (Fig. 1b). Moreover, ES did not affect males, but increased and decreased total ghrelin levels in P9 (condition\*sex\*age:  $F_{1,42} = 26.28$ ,  $p < 0.01$ ; *post-hoc*:  $p = 0.02$ ) and P14 ES-exposed females (*post-hoc*:  $p < 0.01$ ), respectively (Fig. 1b). While AG levels were reduced from P9 to P14 in both sexes (age:  $F_{1,41} = 27.88$ ,  $p < 0.01$ ; Fig. 1c), DAG levels showed similar patterns as total ghrelin, i.e. increased DAG from P9 to P14 in CTL and ES males (age:  $F_{1,34} = 45.98$ ,  $p < 0.01$ ; Fig. 1d), and CTL females (condition\*age:  $F_{1,34} = 12.76$ ,  $p < 0.01$ , *post-hoc*:  $p = 0.09$ ), but not ES females, which was accompanied by decreased DAG in P14 ES-exposed females (condition\*sex\*age,  $F_{1,34} = 8.90$ ,  $p < 0.01$ ; *post-hoc*:  $p < 0.01$ ; Fig. 1d). In addition, ES increased basal insulin levels similarly in both sexes at P9 (males CTL: 784 pg/mL, ES: 1024 pg/mL; females CTL: 529 pg/mL, ES: 985 pg/mL; condition:  $F_{1,36} = 4.04$ ,  $p = 0.052$ ).

At P14, NPY and AgRP immunoreactive fibers were extensively present in the ARH and PVH (Fig. 2a,d). In the ARH, ES resulted in a higher increase in NPY fiber density in females (condition:  $F_{1,17} = 23.33$ ,  $p < 0.01$ , sex:  $F_{1,17} = 7.47$ ,  $p = 0.01$ ; Fig. 2b) and AgRP was unaltered by ES in both sexes (Fig. 2c). Furthermore, ES increased NPY (trend in condition:  $F_{1,16} = 3.69$ ,  $p = 0.07$ ; Fig. 2e) and AgRP fiber density in the PVH (condition:  $F_{1,17} = 4.42$ ,  $p = 0.051$ ; Fig. 2f) similarly in both sexes. Lastly, hypothalamic *GHSR* mRNA expression was unaffected by ES (condition:  $F_{1,23} = 0.88$ ,  $p = 0.36$ ; data not shown).

## 4. Discussion

We here present differential effects of ES on developmental ghrelin levels, depending on the ghrelin form (AG vs DAG), age (P9 vs P14) and sex of the animal, while insulin levels were similarly increased by ES in both sexes at P9. Additionally, ES induced similar changes in hypothalamic NPY/AgRP fiber density in P14 male and female mice, and did not affect hypothalamic *GHSR* mRNA expression.



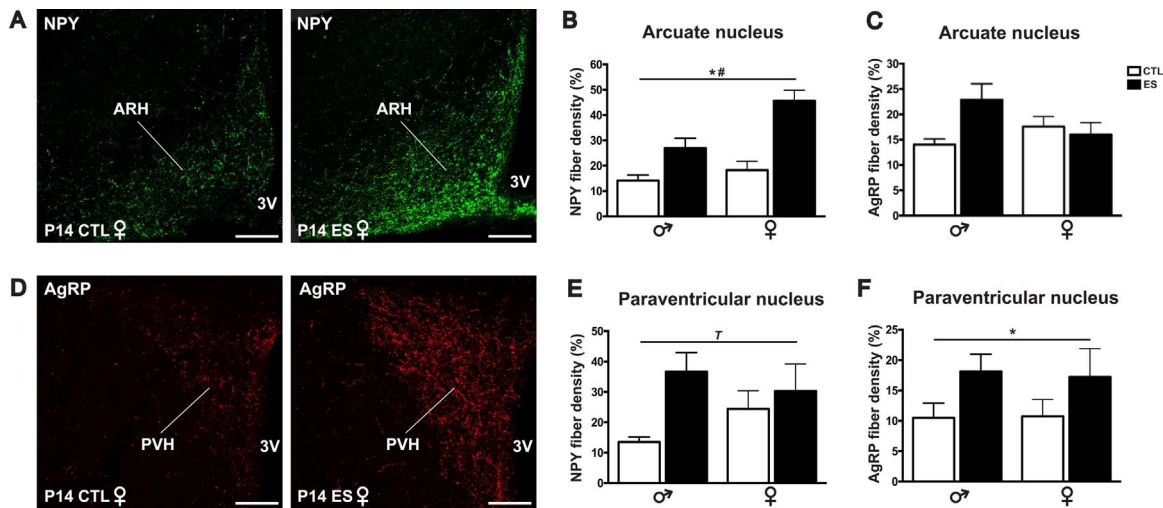
**Fig. 1.** Body weight and ghrelin levels were affected in P9 and P14 males and females after early-life stress exposure. (A) Body weight gain from postnatal day (P)2 to P9 and P9 to P14 in control (CTL) and early-life stress (ES) males and females. (B) Total ghrelin levels, (C) acyl ghrelin and (D) desacyl ghrelin in male and female mice at P9 and P14. Data are presented as mean ± SEM; two-way or three-way ANOVA: interaction between C: condition, S: sex, A: age; \*Games-Howell *post-hoc* test,  $P < 0.05$ .

In contrast to adulthood, ghrelin early in life modulates the development of hypothalamic feeding circuits and does not affect the body weight (Steculorum and Bouret, 2011). Here we observe that ES reduced body weight gain from P2 to P9, but ES-exposed mice grew significantly more between P9 and P14, leading to comparable body weights by the age of P14 in both sexes. Interestingly, when considering ghrelin, ES induced sex-specific changes in total ghrelin and DAG within this short time frame in females only, suggesting that ghrelin may be differentially involved in body weight regulation early in life in males and females. Next to this, our previous findings indicate the possible involvement of mild cold exposure during the ES period, which might possibly influence body weight regulation as well (Yam et al., 2017).

During development, ghrelin rises gradually from P0 to adult-like levels at P14 in rodents, but a lack of such developmental increases in either total ghrelin, AG or DAG has also been reported in male mice

(Collden et al., 2015) and in male and female rats (Sominsky et al., 2017). The increase in total ghrelin (when present) has been mainly attributed to AG, while DAG has so far been only marginally considered. However, here we show that increased total ghrelin from P9 to P14 in both CTL males and females was largely due to increased DAG, with AG being strongly reduced, indicating that DAG is at least dynamically regulated at this stage of life.

Furthermore, while ES has only minimally affected total ghrelin and DAG levels in males, it strongly increased these levels in P9 ES females, without further increasing it by the age of P14. Such sex-specific effects are particularly interesting as ES clearly leads to an increased prevalence of metabolic disorders in females, but not males (Murphy and Loria, 2017). This elevated DAG could thus potentially affect the formation of hypothalamic feeding circuits adversely and in a sex-specific manner, resulting in an increased vulnerability to metabolic disorders in females during adulthood.



**Fig. 2.** Hypothalamic NPY and AgRP fiber density was increased in both P14 males and females after exposure to early stress. (A) Representative confocal images of neuropeptide Y (NPY) immunoreactive fibers in the arcuate nucleus of the hypothalamus (ARH) located next to the third ventricle (3V) in control (CTL) and early-life stress (ES) P14 female mice. (B) NPY and (C) agouti-related peptide (AgRP) fiber density in the ARH. (D) Representative images of AgRP immunoreactive fibers in the paraventricular nucleus of the hypothalamus (PVH) of CTL and ES P14 females. (E) NPY and (F) AgRP expression in the PVH. Data are presented as mean ± SEM; two-way ANOVA: \*main effect of condition, #main effect of sex,  $P < 0.05$ , T: trend in main effect of condition. Scale bars: 100 μm.



The data available to date mostly concern AG and are, in fact, still contradictory as developmental AG can have inhibitory (Steculorum et al., 2015) as well as stimulatory effects (Collden et al., 2015; Schmidt et al., 2006; Steculorum and Bouret, 2011) on hypothalamic feeding circuits. We here show that ES has no significant effects on AG, but decreases DAG levels in P14 ES-exposed females only. This is accompanied by increases of NPY fiber density in the ARH and AgRP in the PVH, and no alterations in hypothalamic *GHSR* mRNA expression in both sexes at P14. Given the recent evidence that DAG is able to activate ARH neurons in adult mice (Fernandez et al., 2016), our findings could suggest an inhibitory role of DAG on the NPY/AgRP circuitry, particularly in females. Moreover, the differential effects of NPY and AgRP in the ARH in ES females might point towards a different sex-specific regulation of these hypothalamic neurons under stressful conditions. Such differential expression of NPY and AgRP has been described previously after stress exposure in adulthood (Kas et al., 2005) or in response to psychostimulants (Crowley et al., 2005). Interestingly, even though not statistically significant with the two-way ANOVA, we observed a sex-specific 63% increase in AgRP-ARH fiber density in ES males only, indicating subtle sex differences at this level as well. Thus, the strong sex-specific ES-induced effects on DAG could possibly play a role in regulating this neurodevelopmental process in a sex-specific manner under stressful circumstances.

Next to the possible involvement of ghrelin, the ES-induced increase in hypothalamic NPY/AgRP fiber density is likely influenced by other hormones as well, possibly in a synergistic fashion. Here we show increases in insulin at P9 in both sexes. There is evidence that insulin during adulthood affects the hypothalamic circuit (Loh et al., 2017), however, its exact action during development still remains to be defined. This first evidence of increased developmental insulin after ES in mice in both sexes is in contrast to the previously described reductions (Maniam et al., 2015b; Mela et al., 2012) or no changes (Maniam et al., 2015a) in insulin of rats during adulthood. This discordance could be age and/or species related. In addition, we have previously shown that ES decreases circulating leptin (Yam et al., 2017) and increases corticosterone (Naninck et al., 2015) similarly in both sexes at P9. As for leptin, there is evidence that it has a modulatory role on the hypothalamic circuits development, even though opposing or no effects have been reported (Ahima and Hileman, 2000; Bouret et al., 2004; Proulx et al., 2002). On the other hand, while there is evidence for the involvement of glucocorticoids (Konno et al., 2008) and adiponectin (Guillod-Maximin et al., 2009) in adulthood, their effects on the hypothalamic circuitry during the developmental period is yet unknown.

It is interesting to note that while ES similarly affects corticosterone, leptin and insulin in both sexes, ghrelin is more prominently affected in females. In contrast, NPY and AgRP are overall similarly affected by ES in both sexes. This could either mean that the ES-induced alteration in hypothalamic fibers is not modulated by ghrelin but is rather under the control of the other hormones mentioned above, and/or that ghrelin might have a (possibly additional) specific modulatory role in females only under this early-life stressful condition.

To the best of our knowledge, we are the first to show differential effects of chronic ES on the different forms of ghrelin, alterations in early insulin levels, and on main components of the hypothalamic feeding circuitry during the developmental period in both sexes. There are powerful examples in which modulating ghrelin or leptin during the early-life period can reverse changes in the hypothalamic feeding circuitry induced by maternal deprivation (Schmidt et al., 2006) or in ob/ob mice (Bouret et al., 2004). This indicates that a more complete understanding of the effects of ES on metabolic hormones and the hypothalamic feeding system may provide attractive future options to possibly prevent the vulnerability to develop metabolic disorders later in life.

## Conflict of interest

All work was conducted in the absence of commercial or financial relationships that could be interpreted as potential conflict of interest. KY is a recipient of a short-term EMBO fellowship. AK is supported by JPI CogniPlast, NWO-Meervoud and the NOW Food, Cognition and Brain grant. P.J.L. is supported by Alzheimer Nederland. S.J.S. is funded by a National Health and Medical Research Council Career Development Fellowship, a Club Melbourne Fellowship and a Brain Foundation Research Gift.

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