Prenatal Glucocorticoid Exposure Modifies Endocrine Function and Behaviour for 3 Generations Following Maternal and Paternal Transmission

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Fetal exposure to high levels of glucocorticoids programs long-term changes in the physiologic stress response and behaviours. However, it is not known whether effects manifest in subsequent generations of offspring following maternal (MT) or paternal (PT) transmission. We treated pregnant guinea pigs with three courses of saline or synthetic glucocorticoid (sGC) at a clinically relevant dose. Altered cortisol response to stress and behaviours transmitted to juvenile female and male F₂ and F₃ offspring from both parental lines. Behavioural effects of sGC in F₁-F₃ PT females associated with altered expression of genes in the prefrontal cortex and hypothalamic paraventricular nucleus (PVN). Exposure to sGC programmed large transgenerational changes in PVN gene expression, including type II diabetes, thermoregulation, and collagen formation gene networks. We demonstrate transgenerational programming to F₃ following antenatal sGC. Transmission is sex- and generation-dependent, occurring through both parental lines. Paternal transmission to F₃ females strongly implicates epigenetic mechanisms of transmission.

Glucocorticoids (GC) are a potent developmental trigger during pregnancy. The late-gestation GC surge promotes rapid maturation of fetal organs, including the lungs and brain¹. However, fetal exposure to high levels of GC prior to the surge, either through maternal stress or treatment with synthetic glucocorticoids (sGC), alters the trajectory of development and programs stress-associated behaviours (e.g. locomotor activity, attention, anxiety) through altered gene expression in the medial prefrontal cortex (mPFC) and hypothalamic-pituitary-adrenal (HPA) axis²-⁴.

Women at risk for preterm birth between 24–34 weeks gestation receive sGC to improve infant respiratory outcomes⁵. While a single course of sGC is currently the gold-standard therapy, during the 1990’s and early 2000’s multiple course treatment with sGC was common⁶,⁷. Antenatal exposure to multiple courses of sGC has been associated with hyperactivity⁸, impaired attention⁹,¹⁰, and neurodevelopmental impairment¹¹ in young children and animals¹². It is imperative that the long-term effects of antenatal exposure to multiple courses of sGC continue to be investigated since the use of a ‘rescue’ (i.e. a second) course of sGC has recently re-introduced the practice of multiple course administration¹³,¹⁴.

While emerging evidence indicates that antenatal exposure to sGC modifies cardiometabolic and endocrine phenotypes across two generations¹⁵-¹⁹, the effects on HPA activity and stress-related behaviours past the second generation (F₃) are not known. Furthermore, only a few studies have investigated programming by antenatal sGC via the paternal line²⁰-²² though there is strong evidence for paternal transmission to F₂ and F₃ following antenatal

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exposure to stressors. We investigated whether antenatal exposure to sGC in the guinea pig can affect HPA activity and stress-related behaviours in juvenile offspring across multiple generations. The guinea pig was chosen as a model since they share similar patterns of brain development and placentation with the human, and the long gestation, approximately 69 days, allows for targeting specific phases of development. Additionally, the primary GC in guinea pigs is cortisol (as in humans), as compared to corticosterone in mice and rats. We focused our investigation on juvenile offspring since previous studies have indicated that prenatal exposure to sGC results in particularly strong neuroendocrine and behavioural effects in children and young animals. We hypothesized that prenatal sGC exposure results in modified: 1) HPA responsiveness to stress; 2) locomotor activity, anxiety-like behaviour, and sensorimotor gating (attention); 3) altered transcription of key regulatory genes in the mPFC; and 4) altered patterns of gene expression in the PVN. These effects would manifest across three generations, be sex-specific, and depend on the route of transmission (maternal vs. paternal). We show that; 1) transgenerational inheritance in mammals via the paternal lineage does occur, and 2) the effects of a clinically relevant dose of antenatal sGC can be transmitted over multiple generations. These findings have major implications for the study of developmental health and disease.

Results

Reproduction. There was no significant effect of sGC treatment on breeding parameters (age at conception—females 110.0 d ± 1.44; males 137.6 d ± 4.5, number of breeding attempts, length of gestation, pregnancy weight gain, litter size and sex ratio) in the F0-F2 pregnancies (data not shown). There was a trend towards exposure to sGC (F0) increasing the length of gestation for the F2 paternal transmission (PT) pregnancy compared to Veh (Veh 68.5 d ± 0.40, sGC 69.6 d ± 0.35; t23 = 2.003; P = 0.053). The breeding paradigm is presented in Fig. 1.

Growth. F1 & F2. There was no significant effect of sGC treatment on body measures (body length, abdominal circumference, head length and circumference), birth weight, or weight gain in F1 or F2 offspring for either transmission route (data not shown).

F3. Exposure to sGC (F0) increased abdominal circumference compared to Veh in PT males on PND 0 and 20 (PND 0: Veh 8.06 cm ± 0.18, sGC 8.23 cm ± 0.27; PND20: Veh 10.75 cm ± 0.25, sGC 11.33 cm ± 0.22; F(1, 23) = 5.012; P < 0.05). Exposure to sGC also reduced adrenal:body-weight ratio for PT females (Veh 2.00e-4 ± 7.41e-6, sGC 1.73e-4 ± 5.85e-6; t16 = 2.804; P < 0.05). There were no other effects of sGC treatment on growth for either transmission route (data not shown).

HPA Function. There was an effect of time as a repeated measure at all ages for all groups (Fig. 2A and B; P < 0.05).

F1. Antenatal treatment with sGC resulted in an increased cortisol response to open field (OF) stress compared with Veh for females on PND 19 (Fig. 2A; AUCF1 = 2.596; P < 0.05). There were no effects of treatment with sGC on HPA response to stress for females on PND 24 or for males (Fig. 2A and B).

Figure 1. Breeding scheme for (A) Veh and (B) sGC groups. Maternal (Mat) and paternal (Pat) lines originate from F1 female and male breeders (respectively). N represents the total number of independent litters for each treatment, breeding line and generation.
F2. Exposure to sGC (F0) resulted in an increased cortisol response for PT females on PND 19 (Fig. 2A; AUCN; $t_{35} = 2.348; P < 0.05$), and a greater total cortisol response to stress for PT females on PND 24 (Fig. 2B; Veh 2441.0 ng/mL ± 194.3, sGC 3143.0 ng/mL ± 247.1; $t_{36} = 2.255; P < 0.05$). There was a trend towards exposure to sGC (F0) reducing the cortisol response to stress compared to Veh for maternal transmission (MT) females on PND 24 ($t_{28} = 2.021; P = 0.053$). There were no effects of sGC exposure on HPA response to stress for F2 MT and PT males (Fig. 2A and B).

F3. There was an interaction between treatment and time on the cortisol response to stress for PT males on PND 24 (Fig. 2B; $F(3, 72) = 3.383; P < 0.05$), and post-hoc analysis revealed increased cortisol at 60 min compared to Veh (Veh 18.48 ng/mL ± 1.74, sGC 28.87 ng/mL ± 3.86; Tukey HSD; $P < 0.05$). There were trends towards exposure to sGC reducing cortisol response to stress compared to Veh for MT males on PND 19 ($t_{19} = 1.906; P = 0.072$) and increasing cortisol response to stress for PT males on PND 24 (Fig. 2B; AUCN; $t_{24} = 2.051; P = 0.051$). There were no effects of sGC exposure on HPA response to stress for MT or PT females (Fig. 2A and B).

**Open Field Behaviour.** F1. Antenatal treatment with sGC resulted in increased total activity in the OF compared to Veh for females (Fig. 3A; $t_{35} = 2.251; P < 0.05$) and males on PND 19 (Fig. 3A; $t_{33} = 2.039; P < 0.05$). There was a trend towards sGC treatment increasing total activity compared to Veh for females on PND 24 (Fig. 3B; $t_{35} = 1.958; P = 0.058$). There were also trends towards treatment with sGC increasing the distance traveled compared to Veh for males on PND 19 (Veh 833.2 cm ± 158.7, sGC 1233.0 cm ± 143.2; $t_{33} = 1.875; P = 0.070$) and females on PND 24 (Veh 582.9 cm ± 117.4, sGC 1213.0 cm ± 243.9; $t_{33} = 1.947; P = 0.058$). There were no effects of sGC treatment on total locomotor activity for males on PND 24 (Fig. 3A and B), distance traveled by females on PND 19 and males on PND 24 (data not shown), or on the percent of time that F1 offspring spent at the perimeter of the OF chamber (data not shown).

F2. For PT females, exposure to sGC (F0) resulted in increased total activity (Fig. 3A; $t_{36} = 2.092; P < 0.05$), increased distance traveled (Veh 797.3 cm ± 130.0, sGC 1196.0 cm ± 146.6; $t_{36} = 2.042; P < 0.05$), and reduced the percent time spent around the perimeter compared to Veh on PND 19 (Veh 96.9% ± 0.92, sGC 88.3% ± 1.66; $U = 38; P < 0.05$). There were no effects of exposure to sGC on total locomotor activity (Fig. 3A and B), distance traveled (data not shown), or the percent time spent around the perimeter (data not shown) for MT offspring, PT females on PND 24, or PT males.
F3. Exposure to sGC (F0) resulted in increased total locomotor activity compared to Veh for PT females on PND 19 (Fig. 3A; \( t_{23} = 2.065; P < 0.05 \)). There was a trend towards sGC increasing the distance traveled compared to Veh for PT females on PND 19 (Veh 668.0 cm ± 145.2, sGC 1173.0 cm ± 196.3; \( t_{23} = 2.038; P = 0.053 \)). Exposure to sGC also reduced the percent of time around the perimeter compared to Veh for MT males on PND 19 (Veh 91.84% ± 3.14, sGC 57.0% ± 10.21; \( U = 22; P < 0.05 \)). There was a trend towards sGC reducing the percent of time around the perimeter compared to Veh for PT females on PND 24 (Veh 96.71% ± 1.51, sGC 92.55% ± 1.38; \( t_{23} = 2.036; P = 0.053 \)). There were no effects of exposure to sGC on total locomotor activity (Fig. 3A and B) or distance traveled (data not shown) for MT offspring, PT males, or PT females on PND 24. There were also no effects of exposure to sGC on the percent of time around the perimeter (data not shown) for MT females, MT males on PND 24, PT females on PND 19, or PT males.

Acoustic Startle Response and Prepulse Inhibition. There was an effect of prepulse as a repeated measure at all prepulse intensities in all groups (Fig. 4B; \( P < 0.05 \)), indicating that percent inhibition increased as a function of prepulse intensity.

F1. There was no significant effect of sGC treatment on the ASR or PPI for F1 offspring (Fig. 4A and B), but there was a trend towards increased ASR compared to Veh for females (Fig. 4A; \( t_{34} = 1.864; P = 0.071 \)).

F2. There were no significant effects of exposure to sGC (F0) on locomotor activity for either transmission route (Fig. 5A). There was a trend towards exposure to sGC increasing activity compared to Veh for MT females (Fig. 5A; \( F_{(1, 9)} = 4.304; P = 0.068 \)).

Locomotor Activity: Home Cage. There was an effect of time of day on locomotor activity in all groups (Fig. 5A; \( P < 0.05 \)).

F1. In males, there was a within treatment effect of time (\( F_{(23, 368)} = 1.97; P < 0.05 \), and post-hoc analysis revealed that treatment with sGC resulted in a 1-hour phase advance in the profile of activity compared to Veh for males from 0900 h–1100 h (Fig. 5A; Tukey HSD; \( P < 0.05 \) at each time point). There was no effect of sGC treatment on locomotor activity for females (Fig. 5A).

F2. There was a significant effect of exposure to sGC (F0) on locomotor activity for either transmission route (Fig. 5A). There was a trend towards exposure to sGC increasing activity compared to Veh for MT females (Fig. 5A; \( F_{(1, 9)} = 4.304; P = 0.068 \)).
F3. There was a significant interaction between treatment and time of day on locomotor activity for MT males (Fig. 5A; F(23, 138) = 2.136; P < 0.05), and post-hoc analysis revealed that exposure to sGC increased locomotor activity at 0800 h compared to Veh (Fig. 5A; Tukey HSD; P < 0.05). There were no other effects of exposure to sGC on locomotor activity for either transmission route (Fig. 5A).

Body Temperature. F1. sGC treatment reduced overall body temperature compared to Veh for males (Fig. 5B; t16 = 2.215; P < 0.05). There was no effect of sGC treatment on overall body temperature for females or on rhythmicity in males or females (Fig. 5B).

F2 & F3. Overall body temperature was reduced by exposure to sGC in F2 MT males (Fig. 5B; t14 = 2.173; P < 0.05) and F2 PT females compared to Veh (Fig. 5B; t18 = 2.449; P < 0.05). There were no other effects of exposure to sGC on overall body temperature or rhythmicity in F2 or in F3 (Fig. 5B).

Medial Prefrontal Cortex: Gene Expression. F1. Antenatal treatment with sGC reduced the expression of Gnb1 mRNA in the F1 female mPFC compared to Veh (Fig. 6A; t8 = 2.885; P < 0.05). There was no effect of sGC treatment on the expression of Mr, Gr, Fkbp5, or Reln mRNA (Fig. 6A).

F2 & F3. In F2, exposure to sGC resulted in increased expression of Reln mRNA in the PT female mPFC compared to Veh (Fig. 6A; t10 = 2.839; P < 0.05). There was no effect of sGC treatment on the expression of Mr, Gr, Fkbp5, or Gnb1 mRNA in F2, or on the expression of any gene in the F3 mPFC (Fig. 6A).

Hypothalamic Paraventricular Nucleus: Gene Expression. F1. 758 genes were differentially expressed following antenatal treatment with sGC in F1 females compared to Veh (Fig. 7A; FDR < 0.05); expression was increased in 597 genes and reduced in 161. Gene set enrichment analysis (GSEA) for differential gene expression between the Veh and sGC groups revealed enrichment of 160 gene sets (Supplementary Table 1; NES > 1.6, FDR < 0.25); 72 gene sets were positively enriched (i.e. increased expression with sGC treatment) and 88 were negatively enriched (i.e. decreased expression). 14 gene sets were associated with known behavioural, metabolic, and molecular effects of sGC treatment (Table 1; NES > 1.6, FDR < 0.25), including type II diabetes mellitus (Fig. 7B; NES > 1.6, FDR < 0.25). A custom HPA Activity gene set (Table 3) was not enriched (Table 1; NES = 0.92, FDR = 0.618), although the expression of Nr3c2 (encoding Mr) was significantly increased; no other classic HPA genes were differentially expressed.

F2 & F3. Exposure (F0) to sGC resulted in the differential expression of 199 genes in F1 (115 increased, 84 reduced) and 42 genes in F3 (11 increased, 31 reduced; Fig. 7A; FDR < 0.05). 11 genes were differentially expressed...
in F1 and F2 (Myo1d, Galk6, Rapgef5, Cdc42ep2, Kcnal1, Anln, Galnt15, Pitx2, Cdh19, Trim66, and Col18a1), 7 in F1 and F3 (Ank1, Kiaa1024, Exph5, St8sia1, Kiaa1217, Ccde109b, and Sptb), and none of the same genes were differentially expressed in F2 and F3 or in all three generations (Fig. 7A). GSEA revealed enrichment of 47 gene sets in F2 (4 positive, 43 negative) and 41 gene sets in F3 (30 positive, 11 negative; Supplementary Data Table 1; NES > 1.6, FDR < 0.25). 5 gene sets in F2 and 6 in F3 were associated with known behavioural, metabolic, and molecular effects of sGC treatment (Table 1; NES > 1.6, FDR < 0.25), including feeding behaviour and immune function (Fig. 7B; NES > 1.6, FDR < 0.25). The custom HPA Activity gene set (Table 3) was not enriched in either F2 or F3 (Table 1; F2 NES = 1.52, FDR = 0.154; F3 NES = 1.06, FDR = 0.409); there was no effect on any individual classic HPA genes.

**Discussion**

We demonstrate that prenatal treatment with sGC results in transmission of altered HPA response to stress, stress-associated behaviours, and gene expression to F1 via maternal and paternal lineages. These effects are sex- and age-dependent and diminish in advancing generations. The strongest HPA and behavioural effects of sGC treatment occur in female offspring from the paternal line, and F1 and F2 offspring are affected to a greater extent than F3 offspring. Molecular analyses suggest that the mPFC and PVN of female PT animals may be involved in the generation-specific effects of sGC treatment on behaviour. Transmission through the paternal line to the third generation of offspring strongly implicates epigenetic mechanisms in the male germ line as the mode for transgenerational transmission.

Consistent with the effects of sGC in children, we demonstrate an enhanced HPA response to stress in F1 juvenile female offspring. Furthermore, we demonstrate maternal and paternal transmission of altered HPA activity to F2 and F3 offspring. While the HPA response to stress is reduced following maternal transmission, we demonstrate paternal transmission of increased HPA response to stress to F2 females and F3 males. An interesting pattern emerges whereby exposure to sGC affects the stress response in F1 females and F1 males regardless of parental lineage. Sex- and generation-specific programming have been described following prenatal sGC and in other transgenerational studies. However, such a complex pattern of effects is novel and suggests a role for sex chromosomes and/or imprinted genes. It is possible that sGC affect the expression of imprinted genes from the X-chromosome and that this contributes to parent-of-origin × offspring effects. In line with this hypothesis, it has been demonstrated that parentally imprinted genes may be involved in mediating the sex-specific effects of high fat diet (increased body size) in F1 female offspring following paternal transmission.

**Figure 5.** (A) Locomotor activity and (B) average body temperature over 24-hour period in the animal's home cage for F1, F2, and F3 female and male MT (Maternal Transmission) and PT (Paternal Transmission) offspring. Veh (Vehicle) open symbols/bars, sGC (synthetic glucocorticoid) closed symbols/bars. (A) X-axis: solid bar indicates lights off, open bar indicates lights on. Data are expressed as mean ± SEM. A significant difference between Veh and sGC groups is represented as follows: *P < 0.05; **P = 0.068. A significant 1-hour phase difference between Veh and Beta groups is represented as follows: —P < 0.05.
Prenatal treatment with sGC programs hyperactivity in children and young animals. We demonstrate hyperactivity in F₁ females and males on PND 19 following antenatal exposure to sGC, and that this treatment results in increased locomotor behaviour in and open field in F₂-F₃ females on PND 19 following paternal transmission. While hyperactivity can be disruptive in modern society, there may be evolutionary advantages that contribute to the conservation and transmission of this phenotype (e.g. increased resource acquisition and parental attention). This is the strongest and most consistent phenotype that we observe and it provides the first evidence for transgenerational transmission (i.e. paternal line to F₃) following antenatal sGC treatment. Furthermore, these findings demonstrate that adaptive stress behaviours can be transmitted across generations.

It is important to note that variability in locomotor activity in the open field can impact the HPA response to stress of the open field. Therefore, since HPA activity was measured after the open field test, we cannot separate any effect that the animal’s activity while in the open field may have had on HPA response. While we observed increased locomotor activity in F₁ and F₂ PT females that coincided with an increased HPA response to stress for these animals, there was no concomitant increase in HPA activity for F₁ males or F₃ PT females. Furthermore, we have previously demonstrated that prenatal exposure to multiple courses of sGC result in increased locomotor activity in PND 10 female guinea pigs, but there was no effect on HPA response to stress. Thus, while the relationship between stress-induced hyperactivity and increased cortisol production requires further investigation it is clear that prenatal exposure to sGC programs both the HPA and behavioural responses to stress, and that these effects manifest across multiple generations. Offspring were tested twice in the open field to determine the response to the combined stress of maternal separation and the open field (PND 19) and to the open-field alone (PND 24; post-weaning). While it is possible that habituation to repeated testing may have influenced the behavior of the animals in the open field on PND 24, any habituation would have occurred across both control and sGC treated animals, allowing for valid comparisons to be made between groups. Furthermore, the data from the PND 19 clearly demonstrate a robust phenotype of HPA and locomotor hyperactivity in response to stress in F₁-F₃ female offspring, PT. It is acknowledged that interactions may occur when repeated/multiple behavioural testing occurs in the same animal. However, this is an inherent limitation of this type of study; it is not ethically acceptable to produce a separate cohort for each individual test.

Treatment with sGC does not result in hyperactivity when F₁ animals are in the home cage (non-stress environment). However, in F₁ males, sGC treatment results in a phase advance of locomotor activity in anticipation of powerful entrainment signals (lights-on and feeding). Similar effects on circadian behaviour have been observed in rats that were exposed to prenatal stress and involve changes in the expression of clock genes. While transgenerational transmission of altered circadian behaviour occurs in F₂ and F₃, the nature of the effect is different.
from earlier generations (i.e. change in amplitude of activity rather than a shift in the profile) and appears to diminish by F3.

Contrary to our hypothesis, treatment with sGC does not affect anxiety-like behaviour or attention in juvenile F1 offspring. It may be that testing in older animals or using alternative tests would reveal an effect of sGC in our model. Therefore, subsequent studies in older animals need to be undertaken. It is important to note that we assessed passive, automatic attentional processes (gating of irrelevant or unnecessary information), whereas other studies assessed active attention. Exposure to sGC (F0) reduces anxiety-like behaviour in the open field for F1-PT females (PT) and F2 males (MT), and reduces attention in F2 females (PT). However, there is a contradictory trend towards increased anxiety-like behaviour in the acoustic startle chamber for F3 males (MT). Divergence in the response to the acoustic startle, and the open field have been reported in animals that display altered HPA activity, and is likely due to the tests provoking different forms of anxiety-like behaviour (unavoidable and avoidable).

Prenatal treatment with sGC reduces body temperature in F1 and F2 males (MT) and F2 females (PT). Similar effects of sGC treatment have been demonstrated in rats and involve impaired thermogenic signaling (thyroid hormone) in the PVN. In the present study, the hypothermic effects of sGC transmit to the next generation through both parental lines. The fact that sGC reduce overall body temperature without affecting rhythmicity suggests that sGC may affect the magnitude of thermogenic signaling pathways (e.g. production of thyroid hormone) rather than circadian or pulsatile signaling.

Antenatal treatment with sGC does not appear to program an increase in HPA response to stress in F1 and F2 females by altering the expression of HPA regulatory genes in the mPFC or PVN. While this was somewhat surprising, it is important to highlight that the mPFC and PVN were isolated with the animals in a basal state, and future studies need to be undertaken to investigate HPA-related gene expression profiles in the stress-activated state. Alternatively, treatment with sGC may increase HPA responsiveness to stress by increasing the sensitivity.
of the PVN to stress signals. In support of this possibility, we identify a gene expression signature indicative of reduced inhibitory gamma-aminobutyric acid (GABA) signaling in the F1 PVN following treatment with sGC.

Molecular analyses in the mPFC reveal patterns of gene expression that correspond to the effects of sGC on stress-related behaviours. The increase in open field locomotor activity in F1-F3 female offspring (PT) is associated with reduced \(Gnb1\) expression (a signal transduction gene involved in regulating locomotor activity\(^{42,43}\)) only in the F1 mPFC. Additionally, exposure to sGC reduces PPI only in F2 female offspring following paternal transmission, and this is associated with increased \(Reln\) (a cortical development gene involved in regulating attention\(^{44}\)) expression in the mPFC of these animals. Clearly, further work is required to better define these associations.

GSEA of RNA-seq data in the PVN reveals patterns of gene expression associated with altered homeostasis in F1-F3 females (PT). While thermogenic signaling is reduced in F1 and F2, body temperature is reduced only in F2; it is likely that the depletion of this gene set in F1 has more to do with ATP synthesis than with thermogenesis. Indeed, many of the downregulated genes in the F1 and F2 PVN are integral components in the respiratory electron transport chain, including \(Ndufs6\) (encodes the first enzyme in the electron transport chain) and \(Cyc1\) (encodes part of the complex that transfers electrons to cytochrome c). These data indicate that prenatal exposure to sGC may also impact oxidative phosphorylation in the brain. This is an important finding that requires further investigation since oxidative phosphorylation is integral to the processing capabilities of the brain\(^{45}\). We also

| Phenotype                      | Gene Set                                      | Type       | Generation | Effect of sGC | Size | NES     | P-Value  | FDR     |
|-------------------------------|-----------------------------------------------|------------|------------|---------------|------|---------|----------|---------|
| HPA Activity                  | STRESS_ASSOCIATED_GENES                       | Custom     | F1         | Positive      | 18   | 0.92    | 0.618    | 0.618   |
|                               |                                               |            | F2         | Positive      | 18   | 1.25    | 0.154    | 0.154   |
|                               |                                               |            | F3         | Negative      | 18   | 1.06    | 0.409    | 0.409   |
| One-Carbon Metabolism and Methylation | S_ADENOSYLMETHIONINE_DEPENDENT_METHYLTRANSFERASE_ACTIVITY | c5         | F1         | Negative      | 18   | 1.60    | 0.019    | 0.119   |
| Metabolic Dysfunction         | KEGG_TYPE_II_DIABETES_MELLITUS                | c2         | F1         | Positive      | 21   | 1.80    | <0.001   | 0.017   |
|                               | FEEDING_BEHAVIOR                              | c5         | F2         | Positive      | 16   | 1.74    | 0.016    | 0.124   |
|                               | DIGESTION                                     | c5         | F3         | Negative      | 17   | 1.75    | 0.003    | 0.571   |
| Thermoregulation              | REACTOME_RESPIRATORY_ELECTRON_TRANSPORT_ATP_SYNTHESIS_BY_CHEMOSOMATIC_COUPLING_AND_HEAT_PRODUCTION_BY_UNCOUPLING_PROTEINS | c2         | F1         | Negative      | 54   | 3.11    | <0.001   | <0.001  |
|                               |                                               |            | F2         | Negative      | 55   | 1.86    | <0.001   | 0.021   |
|                               | REACTOME_COLLAGENFORMATION                    | c2         | F1         | Positive      | 34   | 1.87    | <0.001   | 0.006   |
|                               |                                               |            | F3         | Positive      | 47   | 1.62    | 0.017    | 0.174   |
|                               | EXTRACELLULAR_MATRIX_STRUCTURAL_CONSTITUENT  | c5         | F1         | Positive      | 19   | 1.92    | <0.001   | 0.005   |
|                               | REACTOME_EXTRACELLULAR_MATRIX_ORGANIZATION   | c2         | F1         | Positive      | 44   | 1.78    | 0.001    | 0.017   |
| Immune Function               | IMMUNE_RESPONSE                               | c5         | F3         | Positive      | 107  | 1.89    | <0.001   | 0.162   |
|                               | CELLULAR_DEFENSE_RESPONSE                     | c5         | F3         | Positive      | 21   | 1.87    | 0.002    | 0.116   |
|                               | KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION   | c2         | F1         | Positive      | 100  | 1.77    | <0.001   | 0.028   |
|                               |                                               |            | F2         | Negative      | 103  | 1.66    | <0.001   | 0.070   |
|                               | REACTOMECELL_SURFACEINTERACTIONS_AT_THE_VASCULAR_WALL | c2         | F2         | Negative      | 54   | 1.62    | 0.005    | 0.125   |
|                               |                                               |            | F3         | Positive      | 57   | 1.89    | <0.001   | 0.017   |
| Signaling                     | REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS     | c2         | F2         | Positive      | 77   | 1.85    | <0.001   | 0.106   |
|                               |                                               |            | F3         | Negative      | 79   | 2.07    | <0.001   | 0.001   |

Table 1. PVN Gene Sets Associated with Phenotypes. Gene sets that are enriched in the F1-F3 female PT PVN and associate with offspring phenotypes. Type: collections of gene sets; c5 = gene ontology, c2 = curated gene sets from published data sets. Effect of sGC: describes relationship between treatment with sGC and the expression of genes within a gene set, positive-increased expression, negative-decreased expression. Size: number of genes in the gene set. NES: normalized enrichment score used to compare gene sets of different sizes. FDR: false discovery rate, the probability that a gene set with a specific NES is a false positive.
observe increased expression of circadian gene sets, including clock genes, in F1 females. These data support the hypothesis that antenatal sGC program circadian behaviour by altering the expression of regulatory clock genes (e.g. Clock and Rora). Maintenance of an intact blood-brain barrier (BBB) at the PVN is paramount to its ability to regulate homeostasis. GSEA reveals enrichment in F1 and F3 for gene networks that regulate BBB competency (e.g. collagen formation and extracellular matrix) following antenatal sGC treatment. Together with our previous study in the fetal guinea pig brain46, these findings demonstrate that prenatal sGC promote BBB competency and provide the first evidence that effects transmit to F3.

Conclusions

This study presents the first evidence that prenatal treatment with sGC results in transgenerational paternal trans-
mission of hyperactivity and altered hypothalamic gene expression through three generations of young offspring. Female offspring appear to be more sensitive than male offspring to the programming effects of sGC, which suggests an interaction between sGC and sex hormones or sex-linked genes. Paternal transmission to F3 strongly implicates epigenetic mechanisms in the process of transmission, and small non-coding RNAs likely play a major role. The mechanisms by which sGC program similar phenotypes in F1-F3 differ between generations, but appear to involve altered transcription of genes in mPFC and PVN. Importantly, neither birth weight nor growth was reduced by sGC. Clearly prenatal treatment with sGC results in very long-term programming effects in the life of an individual, and effects reach into subsequent generations. The findings provide new perspectives on potential factors that may contribute to an individual's vulnerability to disease development. Given the re-emergence of antenatal treatment with multiple courses of sGC, it is imperative that prospective and follow-up studies of human cohorts continue in order to determine the long-term effects of sGC in humans.

### Table 2. qRT-PCR Primer Pairs

| Forward | Reverse |
|---------|---------|
| Mr      | CCCCATATGACATCTCTTTAC |
| Gr      | GGCTGGATGTTGTTGTTGTTG |
| Fkbp5   | TGACCTTGTCTGGTTGTTGTTG |
| Gnb1    | GGCAAGGATGTCTTTGTTGTTG |
| Rotn    | GGCAAGGATGTCTTTGTTGTTG |
| Crh     | GGCAAGGATGTCTTTGTTGTTG |
| Avp     | GGCAAGGATGTCTTTGTTGTTG |
| Sdcp2   | GGCAAGGATGTCTTTGTTGTTG |
| Gabrd   | GGCAAGGATGTCTTTGTTGTTG |
| Acta2   | GGCAAGGATGTCTTTGTTGTTG |
| Gapdh   | GGCAAGGATGTCTTTGTTGTTG |

These RNA species are present in sperm/semenal fluid, can recapitulate the programmed phenotype when injected into an embryo, and are present at approximately twice the level on the X-chromosome as compared to autosomes.
### Methods

**Animals and Treatments.** Twelve week-old female Dunkin-Hartley guinea pigs (F0; Charles River; St Constant, QC, Canada) were singly housed within visual, olfactory and auditory contact of other animals. Food (Teklad Global Guinea Pig Diet # 2040; Envigo) and water were available *ad libitum*. Animals were maintained in temperature (23 °C) and humidity controlled rooms on a 12-hour light-dark cycle (lights on 0700 h, off 1900 h).

Pregnant F0 females were injected with three courses of saline (Veh; 0.166 ml/kg; N = 40) or clinically relevant dose of sGC betamethasone (sGC; 1 mg/kg; Betaject phosphate-acetate mix - Sabex, Boucherville, QC, Canada; N = 40) on GD 40 & 41, 50 & 51, 60 & 61 as previously described17. Female and male F1-F3 offspring were randomly assigned (via dice roll) to juvenile or breeding streams. Investigators were not blinded to the group allocation. Offspring were weighed every 10 days from birth until postnatal day (PND) 40, and measures of body size were collected at birth and PND 20; food/water intake were not measured. Animals designated for breeding did not undergo any behavioural testing. Litters were weaned on PND 20 (water and Teklad Guinea Pig Diet were available) and pair-housed in polycarbonate shoebox cages with an age-, sex-, and treatment-matched partner.

To investigate transgenerational maternal (MT) and paternal (PT) transmission, F1 and F2 female and male F1-F3 offspring were bred with naïve animals (males and females, respectively, purchased from Charles River) to create MT and PT lines of F2 and F3 offspring (Fig. 1A and B). F1 and F2 pregnancies were undissected.

**HPA Testing.** The HPA response to stress of open field (OF) exposure (PND 19 and 24; between 0800 h and 1000 h) was measured through salivary cortisol (animals freely chewed on cotton swabs). Samples were collected at 0 (pre-test in home cage), 30, 60, and 120 min. Saliva was stored at −20 °C until measurement of free cortisol by enzyme linked immunosorbent assay (Salimetrics LLC, State College, PA, USA) as previously described57. Analyses of cortisol for a given test (i.e. OF F1) were run in the same assay. Intra- and interassay coefficients of variance were 8.19% and 11.65%, respectively.

**Behavioral Testing.** **Open Field.** Guinea pigs were tested for total locomotor activity, distance traveled, and percent of time spent in the outer zone of the open field (OF; Opto-max animal activity meter; Columbus Instruments, Columbus, OH, USA; clear Plexiglas box, 42.5 × 42.5 × 23 cm) as previously described17,58. Testing occurred for 30 minutes on PND 19 (prior to weaning) and PND 24 (after weaning) between 0800 h and 1000 h, and OF chamber was disinfected between sessions.

**Acoustic Startle/Prepulse Inhibition.** Offspring were tested for acoustic startle response (ASR; a measure of unavoidable anxiety-like behaviour) and prepulse inhibition of the startle response (PPI; sensory-motor gating, passive attention) in the SR-Lab Startle Response System (San Diego Instruments, San Diego, CA, USA; clear Plexiglas tube, 20 cm long × 8.89 cm diameter) as previously described58. Testing occurred for 20 min, including 5 min acclimatization, on PND 23 between 1400 h and 1600 h. Startle chamber was disinfected between sessions.

### Table 3. Custom HPA Activity Gene Set: STRESS_ASSOCIATEDGENES. Expression data for individual genes in the HPA activity gene set, STRESS_ASSOCIATEDGENES, in the F1-F3 female PT PVN. Log, fold-change from Veh. Unadjusted P-value. FDR adjusted.

| Gene   | F1 Log Fold Change | P-Value | FDR  | F2 Log Fold Change | P-Value | FDR  | F3 Log Fold Change | P-Value | FDR |
|--------|--------------------|---------|------|--------------------|---------|------|--------------------|---------|------|
| Arrn   | −0.089             | 0.326   | 0.603| −0.027             | 0.788   | 0.999| 0.103              | 0.056   | 0.636|
| Avp    | −1.016             | 0.026   | 0.161| 0.541              | 0.043   | 0.508| −0.110             | 0.673   | 0.985|
| Bdnf   | −0.082             | 0.607   | 0.812| 0.130              | 0.431   | 0.499| −0.215             | 0.111   | 0.768|
| Clock  | 0.405              | 0.000   | 0.003| 0.092              | 0.344   | 0.999| −0.023             | 0.667   | 0.984|
| Crh    | −0.991             | 0.004   | 0.057| 0.675              | 0.003   | 0.118| −0.075             | 0.719   | 0.991|
| Crhr2  | 0.322              | 0.028   | 0.168| −0.026             | 0.852   | 0.999| −0.039             | 0.481   | 0.967|
| Cry1   | 0.045              | 0.671   | 0.849| 0.011              | 0.924   | 0.999| −0.037             | 0.624   | 0.979|
| Cry2   | −0.019             | 0.843   | 0.934| 0.013              | 0.898   | 0.999| −0.051             | 0.288   | 0.910|
| Fkbp5  | 0.433              | 0.071   | 0.276| 0.115              | 0.555   | 0.999| 0.081              | 0.501   | 0.971|
| Gad1   | 0.362              | 0.058   | 0.247| −0.076             | 0.755   | 0.999| −0.094             | 0.362   | 0.937|
| Gad1b2 | −0.302             | 0.287   | 0.566| −0.287             | 0.241   | 0.982| −0.105             | 0.631   | 0.979|
|Ian    | 0.024              | 0.810   | 0.918| 0.172              | 0.111   | 0.773| −0.030             | 0.605   | 0.978|
|Nfk1   | 0.177              | 0.178   | 0.445| 0.040              | 0.767   | 0.999| −0.157             | 0.084   | 0.711|
|Nr3c1  | 0.070              | 0.518   | 0.756| 0.059              | 0.550   | 0.999| 0.093              | 0.024   | 0.490|
|Nr3c2  | 0.326              | 0.001   | 0.026| −0.088             | 0.432   | 0.999| −0.062             | 0.318   | 0.920|
|Per1   | 0.131              | 0.262   | 0.543| 0.036              | 0.726   | 0.999| −0.010             | 0.901   | 0.996|
|Per2   | 0.293              | 0.025   | 0.156| 0.059              | 0.622   | 0.999| 0.013              | 0.884   | 0.996|
|Trh    | −0.823             | 0.002   | 0.041| 0.264              | 0.163   | 0.883| −0.139             | 0.602   | 0.978|

Table 3. Custom HPA Activity Gene Set: STRESS_ASSOCIATED_GENES. Expression data for individual genes in the HPA activity gene set, STRESS_ASSOCIATED_GENES, in the F1-F3 female PT PVN. Log, fold-change from Veh. Unadjusted P-value. FDR adjusted.
Baseline ASR was determined by exposure to 4 pulses at 120 dB following acclimatization. Testing sessions consisted of the following 60 trials presented in a pseudo-randomized order as previously described.  

**Activity/Body Temperature.** Radiotelemeters (model TA-F40; Data Sciences International, St. Paul, MN, USA) were subcutaneously implanted on PND 33 to monitor locomotor activity and body temperature over a 24-hour period in the animal’s ‘home’ cage. Data were collected (uninterrupted) every 5 min beginning at 0900 h on PND 35, and summed into one-hour bins for analysis.

**Prefrontal Cortex and PVN Gene Expression: Quantitative Real-Time PCR.** Juvenile guinea pigs were weighed and euthanized in the morning (0900–1100 h) by decapitation at PND 40 (43.6 ± 0.45). The brain was removed, weighed, dissected, and frozen on dry ice. As strongest behavioural effects of prenatal sGC were observed in the F1-F2 paternal line females, this group underwent subsequent molecular analysis. The right frontal cortex and hypothalamus from F1-F2 paternal line females were cryosectioned at −20 °C. 1.0 mm diameter punches (Harvard Apparatus Inc., Holliston, MA, USA) of the mPFC cingulate cortex area 1 and infralimbic cortex (Fig. 6B), and the anteromedial hypothalamus, containing the entire PVN, (Fig. 7B) were collected. Other hypothalamic regions adjacent to the PVN may be represented in the anteromedial punches. RNA from punches was extracted using AllPrep Universal Kit (Qiagen). Bioanalyzer (RNA 6000 Pico LabChip, Applied Biosystems); determined all RNA samples had RIN ≥ 7. cDNA was made using SensiFAST cDNA synthesis kit (Bioline, London, England). qRT-PCR was run in triplicate (SensiFAST SYBER HI-ROX 20 μl reaction, Bioline) and quantified by a CFX96 Real-Time System (Bio-Rad). Expression of target mRNA (Table 2) relative to Gapdh housekeeping gene was assessed using the 2−ΔΔCT method.

**PVN RNA-Sequencing.** mRNA libraries were prepared using Illumina TruSeq V2 mRNA enrichment. Samples were sequenced (F1: Veh N = 5, sGC N = 5; F2: Veh N = 5, sGC N = 5; F3: Veh N = 6, sGC N = 6) on Illumina HiSeq. 2500 at 1 × 31 bp by the Donnelly Sequencing Centre. Reads (~45 million reads per sample) were aligned to the *Cavia porcellus* reference genome (>85% alignment, cavPor3.83) with Tuxedo Suit tools accessed through The Galaxy Project. Subsequent analyses were performed in R (version 3.2.3). Gene read counts were determined with Genomic Alignments (version 1.6.3) as previously described. Outliers were removed using Cook’s distance with cut-offs, and data were normalized by residuals with RUVSeq (version 1.4.0). Differential gene expression was assessed using EdgeR’s (version 3.12.1) general linear model likelihood ratio test, and FDR-corrected P < 0.05 was considered significant. Genotype permutations (1000) were computed in GSEA to determine gene set enrichment. Gene sets with FDR ≤ 0.25, P ≤ 0.01, and NES ≥ 1.6 met significance thresholds. Differential expression for 7 candidate genes assessed by qRT-PCR validated the RNA-seq data as there was a strong correlation between RNA-seq and qRT-PCR results (Supplementary Fig. 1; R² = 0.75). Data is available at Gene Expression Omnibus (GEO) repository, accession number GSE85822. Computer code is available upon request.

**Statistical Analyses.** Analyses of all data (except RNA-Seq) were conducted using STATISTICA 12 (StatSoft, Inc., Tulsa, OK, USA). Investigator was blinded to the group allocation at the time of analysis, and un-blinded after completing analyses. All data are expressed as mean ± SEM; significance is defined as P < 0.05, trend is defined as 0.05 ≤ P < 0.075. Data from same-sex littermates were meaned to prevent litter bias. Sample sizes (N) correspond to independent litters, and not to the total number of offspring across all litters. Power analyses based on previous studies determined N ≥ 8 sufficient to account for inter-litter variability and detect effects in the tests performed. For RNA-sequencing, Scotty web tool determined that 4–6 samples per treatment (N = 4–6) with 40–60 million reads per sample allows for detection of 90–96% of genes with ≥ 1.5 fold change in expression with 80% power at P < 0.05. Following our hypotheses, separate analyses were conducted for sex (female and male), breeding line (MT and PT), and generation (F1, F2, and F3). Outliers were identified using the ROUT method in Prism with the Q set to 1%, and data were normalized by residuals with RUVSeq (version 1.4.0). Differential gene expression was assessed as described above.

**Data Availability.** Data is available at Gene Expression Omnibus (GEO) repository, accession number GSE85822. Computer code is available upon request.
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Acknowledgements

We are grateful to Dr. B.Cox for providing advice on bioinformatic analysis. This work was supported by grants from the Canadian Institutes for Health Research (CIHR; MOP-126166). VGM received funding from the Queen Elizabeth II Graduate Scholarships in Science and Technology and the Ontario Graduate Scholarship. AC received a scholarship from Brain Canada and Kids Brain Health Network. The funding source had no involvement in the final design of the study, in the collection, analysis, and interpretation of data, in the writing of the manuscript, or in the decision to submit the article for publication.

Author Contributions

Each of the authors who have contributed to the production of the manuscript, have consented to having their names on the manuscript and approve the final version of the manuscript. Individual contributions are as follows: V.G.M., A.K., and S.G.M. conceived and designed the experiments. V.G.M. and A.K. performed the experiments. V.G.M. and A.C. analyzed the data. A.K., M.S., and S.G.M. provided advice on the data analysis and the manuscript. V.G.M., A.C., and S.G.M. wrote the manuscript.
Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-11635-w

Competing Interests: The authors declare that they have no competing interests.

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