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1 **ANTENATAL DEXAMETHASONE TREATMENT TRANSIENTLY ALTERS DIASTOLIC FUNCTION**
2 **IN THE MOUSE FETAL HEART**

3

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17

18 **ABSTRACT**

19 Endogenous glucocorticoid action is important in the structural and functional maturation of
20 the fetal heart. In fetal mice, although glucocorticoid concentrations are extremely low
21 before E14.5, glucocorticoid receptor (GR) is expressed in the heart from E10.5. To
22 investigate whether activation of cardiac GR prior to E14.5 induces precocious fetal heart
23 maturation, we administered dexamethasone in the drinking water of pregnant dams from
24 E12.5-E15.5. To test direct effects of glucocorticoids upon the cardiovascular system we

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used SMGRKO mice, with *Sm22-Cre*-mediated disruption of GR in cardiomyocytes and vascular smooth muscle. Contrary to expectations, echocardiography showed no advancement of functional maturation of the fetal heart. Moreover, litter size was decreased 2 days following cessation of antenatal glucocorticoid exposure, irrespective of fetal genotype. The myocardial performance index and E/A wave ratio, markers of fetal heart maturation, were not significantly affected by dexamethasone treatment in either genotype. Dexamethasone treatment transiently decreased the myocardial deceleration index (MDI; a marker of diastolic function), in control fetuses at E15.5, with recovery by E17.5, 2 days after cessation of treatment. MDI was lower in SMGRKO than in control fetuses and was unaffected by dexamethasone. The transient decrease in MDI was associated with repression of cardiac GR in control fetuses following dexamethasone treatment. Measurement of glucocorticoid levels in fetal tissue and hypothalamic corticotropin-releasing hormone (*Crh*) mRNA levels suggest complex and differential effects of dexamethasone treatment upon the hypothalamic-pituitary-adrenal axis between genotypes. These data suggest potentially detrimental and direct effects of antenatal glucocorticoid treatment upon fetal heart function.

INTRODUCTION

Levels of glucocorticoid hormones rise markedly shortly prior to birth, to promote fetal organ and tissue maturation in preparation for birth and subsequent postnatal life (Fowden et al., 1998). This is mimicked with the administration of potent synthetic glucocorticoids (betamethasone or dexamethasone) to pregnant women at risk of preterm delivery, in order to mature fetal organs, thereby reducing neonatal morbidity and mortality should

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49 preterm birth ensue (Roberts et al., 2017, Agnew et al., 2018). A great deal is known about
50 the maturational effects that endogenous as well as exogenous glucocorticoids have on the
51 lungs (Bird et al., 2015, Roberts et al., 2017). However, the role of glucocorticoids in the
52 maturation of other fetal organs has not been well characterised and concerns have been
53 raised that non-optimal antental glucocorticoid therapy might increase adverse outcomes,
54 including perinatal death (reviewed, Kemp et al., 2017). The effects of endogenous
55 glucocorticoids upon fetal heart maturation were, until recently, unclear. We demonstrated
56 an important role for endogenous glucocorticoids, acting via the glucocorticoid receptor
57 (GR), in fetal heart maturation. At embryonic day (E) 17.5, global GR knock-out mice show
58 impaired heart structure and function and fail to show the normal maturational changes in
59 late gestation cardiac expression of genes involved in contractile function, calcium handling
60 and energy metabolism (Rog-Zielinska et al., 2013). Tissue-specific knock-out of GR by
61 *smooth muscle protein 22 (SM22)-Cre*-driven recombination showed that most of these
62 maturational effects are attributable to direct actions of GR within cardiomyocytes and/or
63 vascular smooth muscle cells (VSMC) (Rog-Zielinska et al., 2013). Expression profiling and *in*
64 *silico* transcription factor analysis in sheep heart suggest that endogenous glucocorticoids
65 similarly promote the metabolic transitions that occur close to birth in large animals
66 (Richards et al., 2015). In contrast, although a number of animal studies have investigated
67 the effects of fetal exposure to exogenous glucocorticoids upon subsequent heart structure
68 and function in adulthood (termed “fetal programming” (Agnew et al., 2018, Rog-Zielinska
69 et al., 2014)), few have examined the impact of exogenous glucocorticoid administration
70 upon the heart *in utero*, likely to underpin the long-term effects. Studies in fetal sheep
71 hearts have shown that late gestation maternal hypercortisolemia impacts the myocyte
72 transcriptome, with some changes indicative of precocious maturation, but others not

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73 (Richards et al., 2014). However, whether these effects are mediated by alterations in
74 maternal or placental physiology, or reflect direct actions upon fetal cardiomyocytes is
75 unknown. A clear consensus regarding the direct impact of exogenous glucocorticoid upon
76 the fetal heart has yet to emerge.

77

78 Here, we hypothesised that early activation of GR would advance fetal heart maturation and
79 that this would be via direct activation of GR in fetal cardiomyocytes. We tested our
80 hypothesis by administering antenatal dexamethasone in mice, from E12.5 to E15.5 (prior to
81 the natural late gestation peak in endogenous glucocorticoid levels at E16.5 to E17.5). To
82 assess the direct involvement of GR in cardiomyocytes we used SMGRKO mice, with *SM22-*
83 *Cre*-driven deletion of GR in cardiomyocytes and VSMC. The effect on heart function *in utero*
84 was investigated at E15.5 and at E17.5, during and 2 days following cessation of treatment,
85 respectively. Parameters of glucocorticoid action were investigated *ex vivo*, including GR
86 (*Nr3c1*) and GR target genes (*Fkbp5, Kcnj12*) as well as markers of cardiac maturation -
87 genes encoding calcium handling proteins that are indirectly regulated by GR in heart (Rog-
88 Zielinska et al., 2015).

89

90 **MATERIALS AND METHODS**

91 **Animals**

92 All animal experiments were approved by the University of Edinburgh Animal Welfare and
93 Ethical Review Body and were carried out in strict accordance with accepted standards of
94 humane animal care under the auspices of the Animal (Scientific Procedures) Act UK 1986.
95 SMGRKO mice (on a C57Bl/6 genetic background) have been previously described (Rog-
96 Zielinska et al., 2013). They carry a deletion of exon 3 of the *Nr3c1* gene (encoding GR) in

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97 cardiomyocytes and vascular smooth muscle, the result of *Sm22-Cre* mediated
98 recombination of a 'floxed' GR allele ($GR^{fl/fl}$). Experimental SMGRKO fetuses and control
99 littermates were generated by mating male SMGRKO ($GR^{fl/fl}$ $Sm22\text{-}Cre^{+}$) mice with control
100 females ($GR^{fl/fl}$ $Sm22\text{-}Cre^{-}$). The morning of the day the vaginal plug was found was
101 designated E0.5. Singly housed pregnant females received either dexamethasone
102 (100µg/kg/d, dose calculated based on daily weight and water intake measurements per
103 mouse)(Sigma-Aldrich, St Louis, USA) or vehicle (EtOH, approx 0.023% depending on body
104 weight and water consumption) in their drinking water from E12.5 to E15.5. Cre genotyping
105 was carried out on tissue biopsies using primers: 5'-GATCGCTGCCAGGATATACG-3' and 5'-
106 AGGCCAGGTATCTCTGACCA-3'. Fetuses were weighed then killed by decapitation at either
107 E15.5 or E17.5. Fetal heads were rapidly frozen on dry ice for *in situ* mRNA hybridisation. For
108 RNA extraction, fetal hearts were dissected, weighed and rapidly frozen on dry ice. For
109 liquid chromatography mass-spectrometry (LC-MS/MS), fetal and maternal livers were
110 dissected, weighed and rapidly frozen on dry ice. Tissues were stored at -80°C prior to
111 extraction and analysis.
112

113 **High-resolution ultrasound analysis**

114 Fetal cardiac function was assessed at E15.5 or E17.5 using a Visualsonics Vevo 770 High
115 Resolution Ultrasound Scanner. Briefly, pregnant mice were anaesthetised with 2%
116 isoflurane gas, laid supine with all limbs taped to electrocardiogram electrodes to monitor
117 heart rate (~450bpm) and body temperature maintained at 37°C. Fetuses were counted for
118 identification purposes and all were scanned. Abdominal hair was removed using a
119 commercially available depilating cream, then pre-warmed ultrasound gel (Aquasonic 100;
120 Parker Laboratories, Orange, NJ, USA) was applied. A 30 MHz RMV 707B (real-time

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121 microvisualization) transducer was used, with a focal length of 12.7mm and a depth of field
122 view of 20mm. Pulse-wave Doppler was used to measure blood flow across the mitral valve
123 using the apical four chamber view. Vevo 770 image analysis software was used to measure
124 and analyse B-mode images and Doppler waveform traces. Following imaging, the dam was
125 killed by cervical dislocation. Scanned fetuses were excised following identification by
126 validation of position from the scanned images, then weighed, decapitated and tissues
127 collected.
128

129 **RNA analysis**

130 RNA was extracted from fetal hearts by homogenisation in TRIzol (Thermo Fisher Scientific)
131 followed by purification using a Purelink RNA mini kit (Thermo Fisher Scientific). cDNA was
132 synthesised using a QuantiTect Reverse Transcription Kit (Qiagen) then subject to
133 quantitative (q)PCR (in triplicate) using the Roche Lightcycler 480 system with gene-specific
134 primer sets and Universal Probe Library (Roche) (Supplementary Table 1). A standard curve
135 was prepared from pooled cDNA samples. Relative quantification was provided by
136 LightCycler software using the maximum second derivative method. mRNA levels are
137 expressed relative to a combination of the mean of 2 or more of *18s*, *Actb, Hprt* and *Tbp*
138 mRNA levels, used as internal standards (as detailed in the figure legends).
139

140 ***In situ* mRNA hybridisation**

141 Coronal sections (10µm) of fetal heads were cut using a cryostat, collected onto Polysine®
142 slides and stored at −75°C until processing. Corticotropin-releasing hormone (*Crh*) mRNA
143 was measured using a ^{35}S-UTP radio-labelled cRNA probe used extensively by ourselves and
144 others (Welberg et al., 2000, Brunton et al., 2015, Grundwald and Brunton, 2015, Harris et

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145 al., 2001) under conditions optimised in our laboratory. Briefly, ^{35}S-cRNA was synthesized
146 from a linearized pBluescript vector expressing a 518bp *Crh* cDNA fragment (a gift from Dr.
147 Robert Thompson, University of Michigan, Ann Arbor, MI, USA). The plasmid was linearized
148 with *HindIII* or *XbaI* and transcribed from the T7 or T3 promoters, respectively to synthesize
149 the sense and antisense probes. ISH was performed as previously described (Brunton et al.,
150 2009), except that following the RNase A step, slides were washed 3 x 50 minutes in 0.1 x
151 SSC at 60°C. Following the post-hybridization washes, sections were dehydrated in an
152 ascending series of ethanol containing 300mM ammonium acetate, air-dried and then
153 exposed to autoradiographic film (Amersham Hyperfilm MP, GE Healthcare,
154 Buckinghamshire, UK) at room temperature. Films were developed (Ilford Phenisol) and
155 fixed (Ilford Hypam fixer). To optimise exposure times for autoradiographic film, a trial set of
156 slides were exposed to film and developed at 2 day intervals until the exposure time which
157 produced the best signal to noise ratio was identified and this exposure time (6 days) was
158 used for the experimental tissue. Slides were subsequently dipped in autoradiographic
159 emulsion and exposed at 4°C for 5 weeks then developed, counterstained with
160 haematoxylin and eosin and viewed under a light microscope to confirm neuroanatomical
161 localisation of the PVN. CRH mRNA expression in the paraventricular nucleus (PVN) of the
162 hypothalamus was examined bilaterally in 3-6 sections/mouse with measurements taken
163 from the mid-section on which CRH was apparent. Semiquantitative densitometric analysis
164 of autoradiographic films using ImageJ software was used to calculate mean grey density.
165 Sections hybridized with sense probes served as negative controls and showed no signal
166 above background.
167

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168 **Western Blot**

169 Protein was extracted from fetal heart tissue by homogenising in RIPA lysis and extraction
170 buffer, followed by protein quantification using a Pierce BCA assay (both Thermo Fisher
171 Scientific). Samples (20µg protein) were added to NuPAGE Sample Reducing Buffer and
172 NuPAGE LDS Sample Buffer (both Thermo Fisher Scientific), and denatured at 70°C for 10
173 minutes prior to electrophoresis on a NuPAGE Novex 4–12% Bis Tris gel in NuPAGE MES SDS
174 running buffer (Thermo Fisher Scientific). Following protein transfer to a nitrocellulose
175 membrane, non-specific binding was blocked by incubation in 5% blotting-grade blocker
176 (BioRad) in TBS-T for 1 hour. Membranes were incubated with primary antibodies: GR
177 (1:400; G-5: sc-393232, Santa Cruz) and β-tubulin (1:1000; MAB3408, Merck MilliPore)
178 overnight at 4°C. Membranes were then washed and incubated with secondary antibodies
179 IRDye 800CW Goat anti-mouse IgG (1:10000; 926-32210, Licor Biosciences) and IRDye®
180 680RD Goat anti-Mouse IgG (1:10000; 925-68070, Licor Biosciences), followed by
181 quantification of GR protein levels relative to β-tubulin using an Odyssey infrared imaging
182 system (Licor Biosciences).

183

184 **Liquid chromatography-mass spectrometry (LC-MS/MS)**

185 Livers were pooled from all fetuses from a litter to accumulate a weight of approximately
186 100mg tissue (1 sample per litter). Tissues were homogenised in 3ml methanol:water (7:2,
187 v/v) and enriched with 5ng internal standard solution (Epi-corticosterone d4-
188 dexamethasone, d4-cortisol) added to each sample. Homogenates were shaken then
189 centrifuged at 3200g for 45 minutes at 4°C before collecting the supernatant and reducing
190 to dryness using oxygen-free nitrogen at 60°C. For simultaneous quantification of
191 dexamethasone, 6-hydroxydexamethasone, 11-dehydrodexamethasone, corticosterone and

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192 11-dehydrocorticosterone, calibration standards (1mg/ml) of each steroid were diluted to
193 10µg/mL stock in methanol to generate a calibration curve (range, 0.0001-50ng, geometric
194 progressions of 25) enriched with 5ng internal standard solution (Epi-corticosterone d4-
195 dexamethasone, d4-cortisol) and reduced to dryness under oxygen-free nitrogen. Samples
196 and calibration standards were resuspended in 2ml 20% methanol and transferred to Classic
197 Sep-pak columns (2mg, Waters®, Borehamwood, UK) that had been primed with methanol
198 (2ml) and water (2ml) before sample loading. Sep Paks were washed with 2ml water and
199 steroids were eluted with 2ml 100% methanol and collected into a glass vial, before
200 reducing to dryness under oxygen-free nitrogen at 60°C. Samples were resuspended in
201 100µl 70:30 water/acetonitrile for LC-MS/MS analysis.
202
203 Samples were injected (20µl) onto a C18-AR column (ACE Excel 150 x 2.1mm; 2µm, ACT
204 Technologies, Aberdeen, UK) at 40°C using a flow rate of 0.5ml/min, mobile phase A – water
205 0.1% formic acid, B- acetonitrile 0.1% formic acid from 20-90% B over 10 minutes, protected
206 by a Kinetex KrudKatcher® (Phenomenex, Macclesfield, UK), followed by analysis on a
207 QTrap® 5500 LC-MS/MS System (Sciex) or QTrap 6500 Triple Quadrupole, Mass
208 Spectometer (Sciex) system.
209
210 The peak areas of each steroid and each internal standard were integrated using
211 MultiQuant™ version 3.0.8 software (Version number 3.0.8664.0 2015 AB SCIEX with
212 Scheduled MRM™ Algorithm support) (Sciex, 2015). Linear regression analysis of calibration
213 standards, calculated using peak area ratios of steroid of interest to internal standard, was
214 used to determine the concentration of the steroid of interest in the samples. R^2>0.99 was
215 considered acceptable and within each batch of samples the accuracy at the upper and

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216 lower limits were only accepted if accuracy <20%. The amount of steroid was calculated
217 using linear regression analysis of the peak area ratio and was expressed as ng of analyte
218 per g liver.
219

220 **Statistical analysis**

221 All numerical data are presented as means ± SEM, with $p<0.05$ deemed significant.
222 GraphPad Prism 6 software was used for statistical analysis, with unpaired Student's t-test
223 and two-way ANOVA with post-hoc Tukey or Bonferroni tests, as appropriate. All data were
224 subject to Shapiro-Wilk normality testing prior to further analysis.
225

226 **RESULTS**

227 **Dexamethasone treatment reduced fetal number at E17.5, irrespective of genotype with**
228 **no effect on heart weight. Body weight was increased in SMGRKO mice at E17.5.**

229 Dexamethasone treatment from E12.5 to E15.5 had no effect on litter size at E15.5 (Figure
230 1A). We noted that, irrespective of treatment, E15.5 litters contained more SMGRKO than
231 control fetuses (Figure 1B). There was no effect of dexamethasone on fetal weight at E15.5,
232 nor did this differ between genotypes (Table 1), and there was no association between fetal
233 weight and litter size (Supplementary Figure 1A). Heart weight at E15.5 was unaffected by
234 dexamethasone treatment and was similar between control and SMGRKO mice (Table 1). In
235 contrast, 2 days after cessation of dexamethasone treatment, at E17.5, litter size was
236 reduced (Figure 1C). Both genotypes were affected (Figure 1D), suggesting the decrease in
237 litter size is not due to a direct effect of dexamethasone on the fetal heart via GR. Fetal
238 weight did not show any relationship to litter size at E17.5 (Supplementary Figure 1B), nor
239 did we see any direct evidence for an increase in fetal deaths at E17.5 (2 dead/resorbed

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240 fetuses across all 24 litters at E17.5, *versus* 4 across 24 litters at E15.5). However, at E17.5,
241 dexamethasone treated SMGRKO fetuses weighed more than littermate controls and were
242 also heavier than vehicle treated SMGRKO fetuses (Table 1). This is suggestive of oedema in
243 SMGRKO fetuses, as previously identified (Rog-Zielinska et al., 2013), 2 days following
244 cessation of dexamethasone treatment, however, this requires experimental confirmation.
245 There were no differences in fetal heart weights between genotypes or treatment groups at
246 E17.5 (Table 1).
247
248 **Dexamethasone treatment transiently altered cardiac diastolic function at E15.5 in a GR-**
249 **dependent manner, with no effect on systolic function.**
250 Fetal adrenal glucocorticoid synthesis in mice initiates at E14.5 and peaks around E17.5
251 (Michelsohn and Anderson, 1992). To investigate whether antenatal dexamethasone
252 treatment prior to this can advance the maturation of fetal heart function,
253 echocardiography was performed at E15.5 (end of treatment) or at E17.5 (2 days after
254 cessation of treatment). Heart rate was not significantly affected by genotype or treatment
255 at either time point and was in the normal range for these gestational ages (Supplementary
256 Table 2)(Corrigan et al., 2010). There was no difference in the Doppler-derived myocardial
257 performance index (MPI; a measure of combined systolic and diastolic function) between
258 genotypes at either E15.5 or E17.5, nor was it altered by dexamethasone at either time
259 point. There were also no effects of dexamethasone on any of the components of MPI
260 (IVCT, IVRT, ET), although the IVRT showed the expected maturational decrease between
261 E15.5 and E17.5, irrespective of dexamethasone treatment (Supplementary Table 2). The
262 Doppler-derived E/A wave ratio (the ratio of peak blood flow velocity in early 'passive'
263 diastole to peak velocity in late 'active' diastole) was also unaffected by dexamethasone

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264 treatment, nor did it differ between genotypes (Supplementary Table 2). However, at E15.5,
265 compared to control mice, SMGRKO mice showed a lower mitral deceleration index (MDI),
266 calculated by normalising the early filling deceleration time (DT) for peak E wave velocity.
267 This suggests altered diastolic heart function. Moreover, whilst the MDI of SMGRKO fetuses
268 was unaffected following dexamethasone treatment, dexamethasone decreased the MDI in
269 control mice to a level similar to that of SMGRKO mice (Figure 2A), suggesting MDI is
270 responsive to GR activation in cardiomyocytes/VSMC. By E17.5, the MDI of dexamethasone
271 treated control fetuses had recovered to the same level as in vehicle treated controls,
272 although the reduction in the MDI in SMGRKO mice compared to controls persisted (Figure
273 2B).
274

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275 **Calcium-handling genes, markers of fetal heart maturation, are down-regulated by**
276 **dexamethasone treatment.**
277 In their report of the application of high-frequency ultrasound to assess the maturation of
278 the mouse fetal heart, Corrigan et al failed to detect a change in MPI between E16.5 and
279 E18.5 (Corrigan et al., 2010, Rog-Zielinska et al., 2013), suggesting that MPI alone might not
280 be a reliable marker of heart maturation. We therefore examined expression of genes
281 involved in calcium handling that normally increase during fetal heart maturation and which
282 are indirectly induced by GR in fetal cardiomyocytes (Rog-Zielinska et al., 2015). At E15.5, 2-
283 way ANOVA showed a significant effect of dexamethasone in reducing cardiac expression of
284 *Atp2a2, Cacna1c* and *Slc8a1* (encoding SERCA2a, $Ca_v1.2$ and NCX1, respectively); *Ryr2* was
285 unaffected (Supplementary Figure 2A). By E17.5, expression had recovered in
286 dexamethasone-exposed fetuses, with *Cacna1c* even increased in dexamethasone-exposed
287 control fetuses (Supplementary Figure 2B). Overall, these findings are not consistent with an
288 advancement in fetal heart maturation and moreover, suggest that dexamethasone may
289 transiently impair fetal heart function by reducing capacity for calcium handling. This
290 occurred in both genotypes and is therefore independent of GR expression in
291 cardiomyocytes/VSMC.
292

293 **Glucocorticoid signalling in fetal heart may be down-regulated by dexamethasone**
294 **treatment.**
295 The decrease in MDI following dexamethasone treatment in control fetuses at E15.5, to the
296 same level as that in SMGRKO fetal hearts, suggests that dexamethasone may have down-
297 regulated GR signalling in control fetal hearts, causing MDI (a dynamic measure) to be

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298 equivalent to that in the GR-deficient SMGRKO hearts. Consistent with this idea, levels of
299 *Nr3c1* mRNA (encoding GR) in control fetal hearts tended to be reduced (p=0.07) following 3
300 days dexamethasone treatment (Figure 3A). There was also a non-significant trend for
301 down-regulation of GR protein levels in hearts of control fetuses at E15.5 following
302 dexamethasone treatment (Supplementary Figure 3). As expected, *Nr3c1* mRNA levels were
303 lower in SMGRKO fetal hearts at E15.5 compared to control littermates and were unaffected
304 by dexamethasone (Figure 3A). By E17.5, 2 days after cessation of dexamethasone
305 treatment, cardiac *Nr3c1* mRNA levels had largely recovered in control fetuses (Figure 3D).
306 Levels of *Fkbp5* mRNA, a glucocorticoid target gene, did not show the expected increase
307 with dexamethasone treatment in control fetal hearts at E15.5, nor were they different in
308 SMGRKO hearts (Figure 3B). At E17.5, *Fkbp5* mRNA levels remained unaffected by prior
309 dexamethasone treatment (Figure 3E). Similarly, *Kcnj12*, a direct target of GR in fetal
310 cardiomyocytes (Rog-Zielinska et al., 2015), was down-regulated by dexamethasone in
311 control fetuses at E15.5 (Figure 3C), with recovery of normal levels by E17.5 (Figure 3F).
312 These data are consistent with an acute down-regulation of glucocorticoid signalling
313 following 3 days of dexamethasone treatment.
314

315 **Fetal glucocorticoid levels are altered by dexamethasone treatment.**

316 To establish that dexamethasone reached the fetal circulation and to determine effects on
317 endogenous glucocorticoid concentrations, the level of dexamethasone and its major
318 metabolites 6-hydroxydexamethasone and 11-dehydrodexamethasone were measured. We
319 have previously measured corticosterone levels in the mid- and late-gestation fetal heart
320 (Rog-Zielinska et al., 2013), in which the rise in glucocorticoid levels reflected the late-
321 gestation rise in plasma and tissue glucocorticoid levels. Because we had limited amounts of

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322 heart tissue, we reasoned that the concentration of steroids in liver, as an important target
323 of glucocorticoid action as well as steroid and drug metabolism, would be indicative of
324 overall exposure. Fetal livers were pooled from each litter to obtain sufficient sample for
325 mass spectrometry measurement of steroid concentration. The endogenous
326 glucocorticoids, corticosterone and 11-dehydrocorticosterone (11-DHC) were
327 simultaneously measured. At E15.5, dexamethasone was detected in the livers of treated
328 fetuses, though at lower levels than 6-hydroxydexamethasone (Figure 4A, B). 11-
329 dehydrodexamethasone was undetectable (over a dynamic range of assay, 0-10ng). By
330 E17.5, neither dexamethasone nor its metabolites were detectable in fetal livers, nor were
331 they present in vehicle-treated fetuses at either time point (data not shown). Similarly,
332 dexamethasone and 6-hydroxydexamethasone were detected in the livers of
333 dexamethasone-treated pregnant dams at E15.5 (Supplementary Figure 4), but not at E17.5,
334 while 11-dehydrodexamethasone was undetectable (data not shown).
335
336 Consistent with the low glucocorticoid environment of the fetus, corticosterone levels were
337 low in pools of fetal livers at E15.5 and were reduced by dexamethasone treatment (Figure
338 5A). Corticosterone levels remained suppressed in dexamethasone-exposed fetuses at
339 E17.5, 2 days after cessation of treatment (Figure 5C). In contrast, 11-dehydrocorticosterone
340 levels were not significantly affected by dexamethasone treatment at either time point
341 (Figure 5B, D). Corticosterone levels in the liver of the dam were much higher than in the
342 fetus, and although hepatic corticosterone levels in the dam tended to be reduced following
343 dexamethasone treatment, this was not statistically significant (Supplementary Figure 5).
344 Fetal hepatic 11-dehydrocorticosterone concentrations were an order of magnitude lower
345 than corticosterone in the dams and were reduced by dexamethasone-treatment at E15.5.

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346 However, levels had recovered by E17.5. These data could reflect hypothalamic-pituitary-
347 adrenal (HPA) axis suppression, reducing levels of corticosterone and/or 11-
348 dehydrocorticosterone (plasma levels of 11-dehydrocorticosterone increase following HPA
349 axis activation (Kotelevtsev et al., 1997, Harris et al., 2001, Verma et al., 2018)).
350 Alternatively, alterations in absolute or relative activities of maternal or fetal 11β-
351 hydroxysteroid dehydrogenases, which interconvert active and intrinsically inert
352 glucocorticoids (Chapman et al., 2013), could account for the effects of dexamethasone on
353 endogenous glucocorticoid levels. To try to distinguish between these possibilities, we
354 carried out *in situ* mRNA hybridisation to measure levels of *Crh* mRNA in the fetal
355 paraventricular nucleus of the hypothalamus. However, whilst the expected *Crh* down-
356 regulation was seen in dexamethasone-treated SMGRKO fetuses at E15.5, there was no
357 significant effect in control fetuses at this age, nor were there any significant treatment
358 differences between fetuses at E17.5 (Supplementary Figure 6). Thus, the reduced
359 corticosterone levels in dexamethasone-treated fetuses may result from altered placental
360 and/or fetal glucocorticoid metabolism and/or transport. Alternatively, they may be a
361 consequence of changes in pituitary and/or adrenal sensitivities, which were not assessed
362 here.
363
364 **DISCUSSION**
365 Contrary to our hypothesis, administration of dexamethasone from E12.5-E15.5 does not
366 advance fetal heart maturation in mice. MPI, a marker of fetal heart maturity (Corrigan et
367 al., 2010, Rog-Zielinska et al., 2013), was not significantly affected by antenatal
368 dexamethasone, nor were calcium handling genes induced. Heart function was altered
369 though. Dexamethasone transiently decreased the MDI in control fetuses, with recovery by

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370 2 days after cessation of treatment. Other parameters of diastolic function (DT, E/A Doppler
371 velocity ratio, IVRT) were not significantly affected by dexamethasone. There was no effect
372 of dexamethasone on the MDI in SMGRKO fetuses. In an adult population with a high
373 prevalence of hypertension and diabetes but free of prevalent cardiovascular disease, MDI
374 predicted cardiovascular events (Mishra et al., 2007). In another human study, MDI was
375 significantly associated with age and aortic root diameter (Cuspidi et al., 2011). However,
376 the impact of an alteration in MDI during development is unknown, both in the fetus and
377 longer-term in adulthood. Thus, whether the reduction in the MDI in dexamethasone-
378 treated control fetuses is detrimental, beneficial or simply reflects different loading
379 conditions, is currently unclear. Diastolic function is strongly dependent on haemodynamic
380 status and alterations in either preload or afterload affect mitral flow velocity curves
381 (Nishimura et al., 1989a, Nishimura et al., 1989b). The U-shaped relationship between DT
382 and cardiovascular outcomes is strengthened by normalising for the E wave velocity,
383 thereby correcting for loading conditions in calculating the MDI (Chinali et al., 2009, Mishra
384 et al., 2007). Here, DT itself was unaffected by dexamethasone. This suggests that the effect
385 of dexamethasone on the MDI in control fetuses is likely due to alterations in loading,
386 possibly through the well-known increase in fetal blood pressure following antenatal
387 corticosteroid administration (Derks et al., 1997, Bennet et al., 1999). In the surviving
388 dexamethasone-treated control fetuses, MDI was normalised by E17.5. Although diastolic
389 dysfunction in human fetuses with cardiomyopathy is associated with an 8-fold increased
390 risk of fetal mortality compared to systolic dysfunction (Pedra et al., 2002), it is unlikely that
391 any diastolic dysfunction reflected in the lower MDI at E15.5 contributes to the reduced
392 fetal survival at E17.5. Dexamethasone treatment has a similar effect in reducing the
393 number of SMGRKO and control fetuses at E17.5, yet the MDI in SMGRKO mice is

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394 unaffected by dexamethasone. Nevertheless, it is possible that systemic factors, particularly
395 haemodynamic, differentially affect diastolic function in SMGRKO and control fetuses. The
396 increase in wet weight at E17.5 in SMGRKO fetuses with prior dexamethasone treatment is
397 consistent with our previous finding of mild oedema in untreated SMGRKO fetuses at E17.5
398 (Rog-Zielinska et al., 2013) and suggests genotype-specific cardiac and/or haemodynamic
399 effects of dexamethasone. Whether dexamethasone causes or exacerbates oedema in
400 SMGRKO fetuses requires experimental confirmation. SMGRKO mice have normal blood
401 pressure as adults (Richardson et al., 2017). However, in an independent line of mice with
402 GR deletion in VSMC (as in our SMGRKO mice) dexamethasone-induced hypertension is
403 attenuated in adulthood (Goodwin et al., 2008). Whether GR action in VSMC contributes to
404 the fetal heart phenotype observed here remains to be established.
405
406 The lack of difference in MPI between genotypes and with dexamethasone treatment, was
407 surprising, as was the lack of genotype difference in calcium handling gene expression, at
408 least at E17.5. We have previously described an increase in MPI at E17.5 in SMGRKO fetuses
409 compared with controls, and SMGRKO fetuses have lower cardiac expression of *Atp2a2* and
410 *Ryr2* than littermate controls at E17.5 (Rog-Zielinska et al., 2013). The reason we failed to
411 see those differences here is unclear; a reduction in cardiac GR expression in SMGRKO mice
412 was confirmed. However, the SMGRKO mice used here have undergone several generations
413 of breeding since the original line was generated and characterised, and it is possible that
414 compensatory genetic changes have occurred (e.g. due to selection of surviving Cre+ male
415 mice from which to breed), accounting for these findings. It is also possible that the
416 difference in experimental conditions (e.g. daily weighing of the mice or factors unknown to

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417 us that alter haemodynamic status) could account for the discrepancies between this and
418 our previous study. Ongoing and future studies will investigate this.
419
420 Nevertheless, a genotype-specific impact of dexamethasone upon the fetus is evident in this
421 study. Our steroid measurements confirm dexamethasone in fetal livers, albeit at a
422 concentration ~10-fold lower than in the maternal liver. This is consistent with previous
423 reports of a gradient of this magnitude between maternal and fetal serum in the late
424 gestation rat (Varma, 1986). Interestingly, levels of 6-hydroxydexamethasone, an active
425 metabolite of dexamethasone (Ritchie et al., 1992), are at least as high in the fetal liver as in
426 the maternal liver. This relatively high level of 6-hydroxydexamethasone in the fetal liver
427 suggests metabolism of dexamethasone to 6-hydroxydexamethasone by the feto-placental
428 unit. This is the first known example of evidence for 6-hydroxydexamethasone expression in
429 the mouse fetal liver.
430
431 Because, at E15.5, dexamethasone had no effect on the MDI in SMGRKO fetuses, but
432 decreased the MDI in control fetuses (to be the same as in SMGRKO), we hypothesised that
433 GR was down-regulated in cardiomyocytes and/or VSMC of control mice after the 3 days of
434 dexamethasone treatment. Down-regulation of GR in response to dexamethasone
435 treatment is well known (Wilkinson et al., 2018) and lower GR expression in the heart of
436 control animals may explain the lack of dexamethasone effect on *Fkbp5*, a well-known GR
437 target gene as well as *Kcnj12*, a GR target in cardiomyocytes (Rog-Zielinska et al., 2015). Any
438 reduction in glucocorticoid sensitivity due to reduced GR expression is transient, recovering
439 2 days after termination of dexamethasone administration. Genes involved in calcium
440 handling are normally induced following GR activation in cardiomyocytes, albeit by indirect

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441 mechanisms. The transient decrease at E15.5 with recovery by E17.5 is also consistent with
442 down-regulation of GR. However, the decrease in GR that impacts calcium handling is likely
443 not in cardiomyocytes/VSMC, as calcium handling was also affected by dexamethasone in
444 SMGRKO mice. The locus of this regulation of cardiac gene expression by dexamethasone is
445 currently unclear. Dexamethasone treatment also perturbs endogenous fetal glucocorticoid
446 levels, and the effect on the HPA axis appears complex. Prolonged suppression of the fetal
447 HPA axis is suggested by the decrease in fetal corticosterone concentration without a
448 significant change in 11-dehydrocorticosterone following dexamethasone treatment to
449 E15.5 and persisting 2 days after cessation of treatment at E17.5. Whether or not this
450 suppression of the fetal HPA axis persists beyond E17.5 was not investigated here, though
451 antenatal glucocorticoid exposure is known to exert long-term programming effects upon
452 HPA axis activity (Agnew et al., 2018, Rog-Zielinska et al., 2014). Contrary to expectations,
453 *Crh* mRNA was not down-regulated by dexamethasone in control animals, though it was in
454 SMGRKO fetuses at E15.5, with recovery observed by E17.5. Because, for methodological
455 reasons, fetal livers from both genotypes were pooled for steroid measurements, we could
456 not determine whether hepatic corticosterone levels in the SMGRKO and control livers were
457 similarly affected by dexamethasone treatment. Thus, it remains possible that there is a
458 differential effect of dexamethasone upon fetal corticosterone levels in SMGRKO and
459 control fetuses, as well as upon the HPA axis. Whilst speculative, this naturally raises the
460 question of how HPA axis suppression could arise in SMGRKO but not control fetuses. It has
461 been suggested that resistance to negative feedback by glucocorticoids is essential to allow
462 the late gestation increase in glucocorticoid levels (Matthews and Challis, 1996). CRH is not
463 suppressed by antenatal betamethasone treatment in the late gestation sheep fetus,
464 despite reduced cortisol levels (Li et al., 2013). In contrast, in the late gestation guinea pig

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465 fetus, CRH expression is reduced by repeated injection of synthetic glucocorticoids,
466 demonstrating sensitivity to negative feedback (McCabe et al., 2001). Interestingly, in the
467 late gestation fetal sheep hypothalamus, basal CRH synthesis is resistant to
468 hypercortisolaemia, whereas increased CRH synthesis in response to hypoxia is highly
469 sensitive to glucocorticoid negative feedback (Matthews and Challis, 1995b, Matthews and
470 Challis, 1995a). Whether such a mechanism operates in mice requires investigation. CRH
471 synthesis is sensitive to haemodynamic variation in the fetus (Ng, 2000), and conceivably,
472 may be differentially regulated in SMGRKO and control fetuses, if haemodynamic forces
473 differ between the genotypes in response to dexamethasone. It is also possible that sex
474 differences may account for some of the differential effects. For example, the HPA axis of
475 male guinea pig fetuses is more sensitive to negative feedback by maternally administered
476 glucocorticoids at the pituitary level than that of the female fetuses (Owen et al., 2005).
477 Males and females were not distinguished here.
478
479 Dexamethasone reduces survival of both SMGRKO and control fetuses at E17.5 (2 days after
480 cessation of dexamethasone treatment). The cause of death is currently unknown, but
481 plausibly could relate to reduced capacity for calcium handling, which was affected in both
482 genotypes. It is possible that the dexamethasone-related deaths have biased our findings at
483 E17.5, if deaths occurred in more severely impacted fetuses; indeed, a limitation of the
484 current study is that data from all fetuses were included, irrespective of litter size. Thus, the
485 analysis could possibly have been subject to maternal bias. Of note, in a large clinical study
486 in low and mid-income countries, antenatal dexamethasone treatment increased the
487 incidence of neonatal death (Althabe et al., 2015). The risk was greatest in those babies
488 born close to term and importantly, only 16% of the women who recieved antenatal

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489 corticosteroid actually went on to deliver their baby preterm. This raises concerns about
490 possible harmful effects of non-optimally administered antenatal corticosteroids. Our study
491 is consistent with these clinical data and suggest cessation of glucocorticoid treatment
492 confers an increased vulnerability to sudden death. Whilst undoubtedly life saving when
493 appropriately administered, many women administered antenatal corticosteroids in high
494 income countries do not deliver their babies within 1-7 days following treatment (reviewed,
495 Kemp *et al*., 2017). This highlights a need for further research to fully understand the
496 consequences of antenatal glucocorticoid administration, to inform and refine antenatal
497 corticosteroid therapy.

498 In sheep, late gestation maternal hypercortisolemia causes perinatal death (Keller-Wood et
499 al., 2014), associated with cardiac arrhythmias and decreased fetal aortic pressure (Antolic
500 et al., 2018). Thus, there appears an inverted U-shaped relationship between glucocorticoid
501 levels and fetal survival with both glucocorticoid deficiency and glucocorticoid excess
502 reducing perinatal survival. At least some of this effect is likely to be mediated by the
503 complex effects of glucocorticoids on haemodynamic parameters and cardiac function
504 described by us (Richardson et al., 2017, Gray et al., 2016, Rog-Zielinska et al., 2013), and by
505 others (Richards et al., 2014, Reini et al., 2008, Feng et al., 2013, Antolic et al., 2018, Oakley
506 et al., 2013). Unravelling these complex relationships, and the impact of genetic variation in
507 glucocorticoid signalling upon fetal haemodynamic function, will be important for future
508 therapy in preterm birth and in managing the consequences of extreme maternal stress.
509 Effects on placental function were not investigated here. However, placental vascularisation
510 and haemodynamic forces are critical regulators of cardiac maturation (Burton et al., 2016,
511 Thornburg et al., 2010, Morrison et al., 2007). Moreover, recent data in *Hsd11b2*$^{-/-}$ mice
512 suggest pharmacological rescue of impaired placental vasculature also rescues normal fetal

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513 cardiac maturation (Wyrwoll et al., 2016). Future research should address the impact of
514 synthetic glucocorticoid administration upon placental vasculature and haemodynamics, in
515 conjunction with effects on heart.

516 The long-term consequences of the dexamethasone treatment utilised here were not
517 examined. However, in humans and in animals there is an association between excessive
518 glucocorticoid exposure *in utero* and an increased risk of cardiovascular disease in adult
519 offspring (reviewed, (Rog-Zielinska et al., 2014, Agnew et al., 2018)). There are relatively few
520 studies of the effects of antenatal glucocorticoid treatment upon adult cardiovascular
521 disease risk in mice. A previous study in which mice were administered dexamethasone
522 from E12.5 to E15 reported growth restriction and a trend for decreased heart weight at
523 E14.5, with catch up by E17.5. As adults, the dexamethasone-exposed mice had normal
524 hearts, but increased blood pressure and pulse pressure compared to controls (O'Sullivan et
525 al., 2013). Mice are born relatively immature compared to humans. However, the scant
526 evidence available suggests that early postnatal cardiomyocyte maturation, including
527 capacity for proliferation and heart repair, is similar in mice and humans. In both humans
528 and mice there is a period in which cardiomyocytes continue to proliferate after birth (albeit
529 possibly over a longer period in humans than the 1 week postnatally in mice) (Porrello et al.,
530 2011, Mollova et al., 2013, Bergmann et al., 2015, Haubner et al., 2015, Yutzey, 2017).
531 Whether mid to late gestation cardiomyocyte maturation is similar in humans and mice is
532 currently unknown. Our data, however, suggest that even transient effects of antenatal
533 dexamethasone can interfere with the normal trajectory of cardiac maturation, possibly
534 through altered haemodynamics, known to be affected by antenatal dexamethasone in
535 humans (Mulder et al., 2009), thus potentially impacting on adult cardiovascular health.
536 Thus, whilst antenatal glucocorticoids are undoubtedly of clinical benefit in infants born

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537 preterm, there may be detrimental effects on the heart and other organs, as seen here in an
538 animal model. Future studies should establish the short as well as long-term effects of
539 antenatal corticosteroid therapy on human heart. The benefit *versus* risk balance must be
540 carefully considered by clinicians before administering antenatal glucocorticoids. Further
541 research is needed to optimize the timing and dosage to provide the most beneficial
542 outcome overall.

543

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549

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554

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556 Designed research: E.J.A, G.A.G, K.E.C. Execution: E.J.A, A.G.B, R.V.R, H.M, A.J.W.T, K.S, G.J,
557 N.Z.M.H, P.B. Interpretation: E.J.A, A.J.W.T, K.S, G.J, N.Z.M.H, C.M.M, P.B, G.A.G, K.E.C.
558 Manuscript preparation: E.J.A, K.E.C.

559

560 **DECLARATION OF INTEREST**

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561 None of the authors has any conflicts of interest to declare.

562

563 **REFERENCES**

564 AGNEW, E. J., IVY, J. R., STOCK, S. J. & CHAPMAN, K. E. 2018. Glucocorticoids, antenatal
565 corticosteroid therapy and fetal heart maturation. *J Mol Endocrinol,* 61**,** R61-R73.

566 ALTHABE, F., BELIZAN, J. M., MCCLURE, E. M., HEMINGWAY-FODAY, J., BERRUETA, M., MAZZONI, A.,
567 CIGANDA, A., GOUDAR, S. S., KODKANY, B. S., MAHANTSHETTI, N. S., *et al.* 2015. A
568 population-based, multifaceted strategy to implement antenatal corticosteroid treatment
569 versus standard care for the reduction of neonatal mortality due to preterm birth in low-
570 income and middle-income countries: the ACT cluster-randomised trial. *Lancet,* 385**,** 629-39.

571 ANTOLIC, A., WOOD, C. E. & KELLER-WOOD, M. 2018. Chronic maternal hypercortisolemia in late
572 gestation alters fetal cardiac function at birth. *Am J Physiol Regul Integr Comp Physiol,* 314**,**
573 R342-R352.

574 BENNET, L., KOZUMA, S., MCGARRIGLE, H. H. & HANSON, M. A. 1999. Temporal changes in fetal
575 cardiovascular, behavioural, metabolic and endocrine responses to maternally administered
576 dexamethasone in the late gestation fetal sheep. *Br J Obstet Gynaecol,* 106**,** 331-339.

577 BERGMANN, O., ZDUNEK, S., FELKER, A., SALEHPOUR, M., ALKASS, K., BERNARD, S., SJOSTROM, S. L.,
578 SZEWCZYKOWSKA, M., JACKOWSKA, T., DOS REMEDIOS, C. *et al.* 2015. Dynamics of Cell
579 Generation and Turnover in the Human Heart. *Cell,* 161**,** 1566-1575.

580 BIRD, A. D., MCDOUGALL, A. R., SEOW, B., HOOPER, S. B. & COLE, T. J. 2015. Glucocorticoid
581 regulation of lung development: Lessons learned from conditional GR knockout mice. *Mol*
582 *Endocrinol,* 29**,** 158-171.

583 BRUNTON, P. J., DONADIO, M. V., YAO, S. T., GREENWOOD, M., SECKL, J. R., MURPHY, D. & RUSSELL,
584 J. A. 2015. 5α-Reduced neurosteroids sex-dependently reverse central prenatal
585 programming of neuroendocrine stress responses in rats. *J Neurosci,* 35**,** 666-677.

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586 BRUNTON, P. J., MCKAY, A. J., OCHEDALSKI, T., PIASTOWSKA, A., REBAS, E., LACHOWICZ, A. &
587 RUSSELL, J. A. 2009. Central opioid inhibition of neuroendocrine stress responses in
588 pregnancy in the rat is induced by the neurosteroid allopregnanolone. *J Neurosci,* 29**,** 6449-
589 6460.
590 BURTON, G. J., FOWDEN, A. L. & THORNBURG, K. L. 2016. Placental Origins of Chronic Disease.
591 *Physiol Rev,* 96**,** 1509-1565.
592 CHAPMAN, K. E., HOLMES, M. C. & SECKL, J. R. 2013. 11β-hydroxysteroid dehydrogenases:
593 intracellular gate-keepers of tissue glucocorticoid action. *Physiol Rev,* 93**,** 1139-1206.
594 CHINALI, M., AURIGEMMA, G. P., DE SIMONE, G., MISHRA, R. K., GERDTS, E., WACHTELL, K., BOMAN,
595 K., DAHLOF, B. & DEVEREUX, R. B. 2009. Mitral E wave deceleration time to peak E velocity
596 ratio and cardiovascular outcome in hypertensive patients during antihypertensive
597 treatment (from the LIFE echo-substudy). *Am J Cardiol,* 104**,** 1098-1104.
598 CORRIGAN, N., BRAZIL, D. P. & MCAULIFFE, F. M. 2010. High-frequency ultrasound assessment of the
599 murine heart from embryo through to juvenile. *Reprod Sci,* 17**,** 147-157.
600 CUSPIDI, C., NEGRI, F., SALA, C. & MANCIA, G. 2011. Mitral deceleration index is associated with
601 aortic root dilatation and not to biventricular structural changes in essential hypertension.
602 *Blood Press,* 20**,** 190-195.
603 DERKS, J. B., GIUSSANI, D. A., JENKINS, S. L., WENTWORTH, R. A., VISSER, G. H., PADBURY, J. F. &
604 NATHANIELSZ, P. W. 1997. A comparative study of cardiovascular, endocrine and
605 behavioural effects of betamethasone and dexamethasone administration to fetal sheep. *J*
606 *Physiol,* 499**,** 217-226.
607 FENG, X., REINI, S., RICHARDS, E., WOOD, C. E. & KELLER-WOOD, M. 2013. Cortisol stimulates
608 proliferation and apoptosis in the late gestation fetal heart: differential effects of
609 mineralocorticoid and glucocorticoid receptors. *Am J Physiol Regul Integr Comp Physiol,* 305**,**
610 343-350.

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611 FOWDEN, A. L., LI, J. & FORHEAD, A. J. 1998. Glucocorticoids and the preparation for life after birth:
612 are there long-term consequences of the life insurance? *Proc Nutr Soc,* 57**,** 113-122.
613 GOODWIN, J. E., ZHANG, J. & GELLER, D. S. 2008. A critical role for vascular smooth muscle in acute
614 glucocorticoid-induced hypertension. *J Am Soc Nephrol,* 19**,** 1291-1299.
615 GRAY, G. A., WHITE, C. I., CASTELLAN, R. F., MCSWEENEY, S. J. & CHAPMAN, K. 2017. Getting to the
616 heart of glucocorticoid regeneration: 11β-HSD1 and the myocardium. *J Mol Endocrinol*, 58,
617 R1-R13.
618 GRUNDWALD, N. J. & BRUNTON, P. J. 2015. Prenatal stress programs neuroendocrine stress
619 responses and affective behaviors in second generation rats in a sex-dependent manner.
620 *Psychoneuroendocrinology,* 62**,** 204-216.
621 HARRIS, H. J., KOTELEVTSEV, Y., MULLINS, J. J., SECKL, J. R. & HOLMES, M. C. 2001. Intracellular
622 regeneration of glucocorticoids by 11β-hydroxysteroid dehydrogenase (11β-HSD)-1 plays a
623 key role in regulation of the hypothalamic-pituitary-adrenal axis: analysis of 11β-HSD-1
624 deficient mice. *Endocrinology,* 142**,** 114-120.
625 HAUBNER, B. J., SCHNEIDER, J., SCHWEIGMANN, U. U., SCHUETZ, T., DICHTL, W., VELIK-SALCHNER,
626 C., STEIN, J. I. & PENNINGER, J. M. 2016. Functional recovery of a human neonatal heart
627 after severe myocardial infarction. *Circ Res*, 118, 216-221.
628 KELLER-WOOD, M., FENG, X., WOOD, C. E., RICHARDS, E., ANTHONY, R. V., DAHL, G. E. & TAO, S.
629 2014. Elevated maternal cortisol leads to relative maternal hyperglycemia and increased
630 stillbirth in ovine pregnancy. *Am J Physiol Regul Integr Comp Physiol,* 307**,** R405-R413.
631 KEMP, M. W., JOBE, A. H., USUDA, H., NATHANIELSZ, P. W., LI, C., KUO, A., HUBER, H. F., CLARKE, G.
632 D., SAITO, M., NEWNHAM, J. P. *et al.* 2018. Efficacy and safety of antenatal steroids. *Am J*
633 *Physiol Regul Integr Comp Physiol,* 315**,** R825-R839.

634 KOTELEVTSEV, Y., HOLMES, M. C., BURCHELL, A., HOUSTON, P. M., SCHMOLL, D., JAMIESON, P.,
635 BEST, R., BROWN, R., EDWARDS, C. R. W., SECKL, J. R. *et al.* 1997. 11β-hydroxysteroid

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636 dehydrogenase type 1 knockout mice show attenuated glucocorticoid inducible responses
637 and resist hyperglycaemia on obesity or stress. *Proc Natl Acad Sci USA,* 94**,** 14924-14929.
638 LI, S., MOSS, T. J., NITSOS, I., MATTHEWS, S. G., CHALLIS, J. R., NEWNHAM, J. P. & SLOBODA, D. M.
639 2013. The impact of maternal synthetic glucocorticoid administration in late pregnancy on
640 fetal and early neonatal hypothalamic-pituitary-adrenal axes regulatory genes is dependent
641 upon dose and gestational age at exposure. *J Dev Orig Health Dis,* 4**,** 77-89.
642 MATTHEWS, S. G. & CHALLIS, J. R. 1995a. Levels of pro-opiomelanocortin and prolactin mRNA in the
643 fetal sheep pituitary following hypoxaemia and glucocorticoid treatment in late gestation. *J*
644 *Endocrinol,* 147**,** 139-146.
645 MATTHEWS, S. G. & CHALLIS, J. R. 1995b. Regulation of CRH and AVP mRNA in the developing ovine
646 hypothalamus: effects of stress and glucocorticoids. *Am J Physiol,* 268**,** E1096-1107.
647 MATTHEWS, S. G. & CHALLIS, J. R. 1996. Regulation of the hypothalamo-pituitary-adrenocortical axis
648 in fetal sheep. *Trends Endocrinol Metab,* 7**,** 239-246.
649 MCCABE, L., MARASH, D., LI, A. & MATTHEWS, S. G. 2001. Repeated antenatal glucocorticoid
650 treatment decreases hypothalamic corticotropin releasing hormone mRNA but not
651 corticosteroid receptor mRNA expression in the fetal guinea-pig brain. *J Neuroendocrinol,*
652 13**,** 425-431.
653 MICHELSOHN, A. M. & ANDERSON, D. J. 1992. Changes in Competence Determine the Timing of 2
654 Sequential Glucocorticoid Effects On Sympathoadrenal Progenitors. *Neuron,* 8**,** 589-604.
655 MISHRA, R. K., GALLOWAY, J. M., LEE, E. T., BEST, L. G., RUSSELL, M., ROMAN, M. J. & DEVEREUX, R.
656 B. 2007. The ratio of mitral deceleration time to E-wave velocity and mitral deceleration
657 slope outperform deceleration time alone in predicting cardiovascular outcomes: the Strong
658 Heart Study. *J Am Soc Echocardiogr,* 20**,** 1300-1306.
659 MOLLOVA, M., BERSELL, K., WALSH, S., SAVLA, J., DAS, L. T., PARK, S. Y., SILBERSTEIN, L. E., DOS
660 REMEDIOS, C. G., GRAHAM, D., COLAN, S. *et al.* 2013. Cardiomyocyte proliferation
661 contributes to heart growth in young humans. *Proc Natl Acad Sci U S A,* 110**,** 1446-1451.

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Accepted Manuscript published as JOE-18-0666.R2. Accepted for publication: 23-Apr-2019

662 MORRISON, J. L., BOTTING, K. J., DYER, J. L., WILLIAMS, S. J., THORNBURG, K. L. & MCMILLEN, I. C.
663 2007. Restriction of placental function alters heart development in the sheep fetus. *Am J*
664 *Physiol Regul Integr Comp Physiol,* 293**,** R306-313.
665 MULDER, E. J., DE HEUS, R. & VISSER, G. H. 2009. Antenatal corticosteroid therapy: short-term
666 effects on fetal behaviour and haemodynamics. *Semin Fetal Neonatal Med,* 14**,** 151-156.
667 NG, P. C. 2000. The fetal and neonatal hypothalamic-pituitary-adrenal axis. *Arch Dis Child Fetal*
668 *Neonatal Ed,* 82**,** F250-254.
669 NISHIMURA, R. A., ABEL, M. D., HATLE, L. K., HOLMES, D. R., JR., HOUSMANS, P. R., RITMAN, E. L. &
670 TAJIK, A. J. 1989a. Significance of Doppler indices of diastolic filling of the left ventricle:
671 comparison with invasive hemodynamics in a canine model. *Am Heart J,* 118**,** 1248-1258.
672 NISHIMURA, R. A., ABEL, M. D., HOUSMANS, P. R., WARNES, C. A. & TAJIK, A. J. 1989b. Mitral flow
673 velocity curves as a function of different loading conditions: evaluation by intraoperative
674 transesophageal Doppler echocardiography. *J Am Soc Echocardiogr,* 2**,** 79-87.
675 O'SULLIVAN, L., CUFFE, J. S., PARAVICINI, T. M., CAMPBELL, S., DICKINSON, H., SINGH, R. R.,
676 GEZMISH, O., BLACK, M. J. & MORITZ, K. M. 2013. Prenatal exposure to dexamethasone in
677 the mouse alters cardiac growth patterns and increases pulse pressure in aged male
678 offspring. *PLoS One,* 8**,** e69149.
679 OAKLEY, R. H., REN, R., CRUZ-TOPETE, D., BIRD, G. S., MYERS, P. H., BOYLE, M. C., SCHNEIDER, M. D.,
680 WILLIS, M. S. & CIDLOWSKI, J. A. 2013. Essential role of stress hormone signaling in
681 cardiomyocytes for the prevention of heart disease. *Proc Natl Acad Sci U S A,* 110**,** 17035-
682 17040.
683 OWEN, D., BANJANIN, S., GIDREWICZ, D., MCCABE, L. & MATTHEWS, S. G. 2005. Central regulation of
684 the hypothalamic-pituitary-adrenal axis during fetal development in the Guinea-pig. *J*
685 *Neuroendocrinol,* 17**,** 220-6.

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686 PEDRA, S. R., SMALLHORN, J. F., RYAN, G., CHITAYAT, D., TAYLOR, G. P., KHAN, R., ABDOLELL, M. &
687 HORNBERGER, L. K. 2002. Fetal cardiomyopathies: pathogenic mechanisms, hemodynamic
688 findings, and clinical outcome. *Circulation,* 106**,** 585-591.
689 PORRELLO, E. R., MAHMOUD, A. I., SIMPSON, E., HILL, J. A., RICHARDSON, J. A., OLSON, E. N. &
690 SADEK, H. A. 2011. Transient regenerative potential of the neonatal mouse heart. *Science,*
691 331**,** 1078-1080.
692 REINI, S. A., DUTTA, G., WOOD, C. E. & KELLER-WOOD, M. 2008. Cardiac corticosteroid receptors
693 mediate the enlargement of the ovine fetal heart induced by chronic increases in maternal
694 cortisol. *J Endocrinol,* 198**,** 419-427.
695 RICHARDS, E. M., RABAGLINO, M. B., ANTOLIC, A., WOOD, C. E. & KELLER-WOOD, M. 2015. Patterns
696 of gene expression in the sheep heart during the perinatal period revealed by transcriptomic
697 modeling. *Physiol Genomics,* 47**,** 407-419.
698 RICHARDS, E. M., WOOD, C. E., RABAGLINO, M. B., ANTOLIC, A. & KELLER-WOOD, M. 2014.
699 Mechanisms for the adverse effects of late gestational increases in maternal cortisol on the
700 heart revealed by transcriptomic analyses of the fetal septum. *Physiol Genomics,* 46**,** 547-
701 559.
702 RICHARDSON, R., BATCHEN, E., THOMSON, A., DARROCH, R., PAN, X., ROG-ZIELINSKA, E.,
703 WYRZYKOWSKA, W., SCULLION, K., AL-DUJAILI, E. A., DIAZ, M., *et al.* 2017. Glucocorticoid
704 receptor alters isovolumetric contraction and restrains cardiac fibrosis. *J Endocrinol,* 232**,**
705 437-450.
706 RITCHIE, J. C., OWENS, M. J., MAYER, H., WATSON, J. T., KILTS, C. & CARROLL, B. J. 1992. Preliminary
707 studies of 6β-hydroxydexamethasone and its importance in the DST. *Biol Psychiatry,* 32**,** 825-
708 833.
709 ROBERTS, D., BROWN, J., MEDLEY, N. & DALZIEL, S. R. 2017. Antenatal corticosteroids for
710 accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database*
711 *Syst Rev,* 3**,** CD004454.

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Accepted Manuscript published as JOE-18-0666.R2. Accepted for publication: 23-Apr-2019

712 ROG-ZIELINSKA, E. A., CRAIG, M. A., MANNING, J. R., RICHARDSON, R. V., GOWANS, G. J., DUNBAR,
713 D. R., GHARBI, K., KENYON, C. J., HOLMES, M. C., HARDIE, D. G. *et al*. Glucocorticoids
714 promote structural and functional maturation of foetal cardiomyocytes: a role for PGC-1α.
715 *Cell Death Differ,* 22**,** 1106-1116.
716 ROG-ZIELINSKA, E. A., RICHARDSON, R. V., DENVIR, M. A. & CHAPMAN, K. E. 2014. Glucocorticoids
717 and foetal heart maturation; implications for prematurity and foetal programming. *J Mol*
718 *Endocrinol,* 52**,** R125-135.
719 ROG-ZIELINSKA, E. A., THOMSON, A., KENYON, C. J., BROWNSTEIN, D. G., MORAN, C. M., SZUMSKA,
720 D., MICHAILIDOU, Z., RICHARDSON, J., OWEN, E., WATT, A. *et al.* 2013. Glucocorticoid
721 receptor is required for fetal heart maturation. *Hum Mol Genet,* 22**,** 3269-3282.
722 THORNBURG, K. L., O'TIERNEY, P. F. & LOUEY, S. 2010. Review: The placenta is a programming agent
723 for cardiovascular disease. *Placenta,* 31 Suppl**,** S54-59.
724 VARMA, D. R. 1986. Investigation of the maternal to foetal serum concentration gradient of
725 dexamethasone in the rat. *Br J Pharmacol,* 88**,** 815-820.
726 VERMA, M., KIPARI, T. M. J., ZHANG, Z., MAN, T. Y., FORSTER, T., HOMER, N. Z. M., SECKL, J. R.,
727 HOLMES, M. C. & CHAPMAN, K. E. 2018. 11β-hydroxysteroid dehydrogenase-1 deficiency
728 alters brain energy metabolism in acute systemic inflammation. *Brain Behav Immun,* 69**,**
729 223-234.
730 WELBERG, L. A. M., SECKL, J. R. & HOLMES, M. C. 2000. Inhibition of 11β-hydroxysteroid
731 dehydrogenase, the foeto- placental barrier to maternal glucocorticoids, permanently
732 programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring. *Eur J*
733 *Neurosci,* 12**,** 1047-1054.
734 WILKINSON, L., VERHOOG, N. J. D. & LOUW, A. 2018. Disease and treatment associated acquired
735 glucocorticoid resistance. *Endocr Connect*.
736 WYRWOLL, C. S., NOBLE, J., THOMSON, A., TESIC, D., MILLER, M. R., ROG-ZIELINSKA, E. A., MORAN,
737 C. M., SECKL, J. R., CHAPMAN, K. E. & HOLMES, M. C. 2016. Pravastatin ameliorates placental

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738 vascular defects, fetal growth, and cardiac function in a model of glucocorticoid excess. *Proc*
739 *Natl Acad Sci U S A,* 113**,** 6265-6270.
740 YUTZEY, K. E. 2017. Cardiomyocyte Proliferation: Teaching an Old Dogma New Tricks. *Circ Res,* 120**,**
741 627-629.
742

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1 **FIGURE LEGENDS**

2

3 **Figure 1: Dexamethasone treatment reduces fetal number and litter size at E17.5 without**
4 **affecting either at E15.5.**

5 Dexamethasone (Dex, stippled bars: 100μg/kg/day) or vehicle (Veh, white bars) was
6 administered to pregnant dams in their drinking water from E12.5 to E15.5. Individual data
7 points represent mean values per litter. Data are means ± SEM and were analysed by
8 Student's *t*-test (A, C) or 2-way ANOVA with post-hoc Tukey tests (B, D). Significant effect of
9 treatment, *p<0.05, **p<0.01; significant effect of genotype, #p<0.05, n=12-13 litters per
10 group.

11

12 **Figure 2: Dexamethasone transiently reduces the mitral deceleration index, dependent on**
13 **GR.**

14 Dexamethasone (100μg/kg/day; stippled bars) or vehicle (Veh, white bars) was
15 administered to pregnant dams from E12.5 to E15.5. Mitral deceleration index (MDI) was
16 measured by *in vivo* ultrasound imaging at (A) E15.5 and (B) E17.5. Data are means ± SEM
17 and were analysed by 2-way ANOVA with post-hoc Tukey tests: significant effect of
18 treatment, *p<0.05; significant effect of genotype, #p<0.05, n=8-28 fetuses from 5-6 litters
19 per group.

20

21 **Figure 3: Glucocorticoid signalling in hearts of control fetuses is down-regulated by**
22 **dexamethasone at E15.5.**

23 Dexamethasone (100μg/kg/day, stippled bars) or vehicle (Veh, white bars) was
24 administered to pregnant dams in their drinking water from E12.5 to E15.5. qRT-PCR

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25 measurements at E15.5 of cardiac levels of (A) *Nr3c1* mRNA, (B) *Fkpb5* mRNA, (C) *Kcnj12*
26 mRNA, all relative to the mean of *Actb*, *Hprt* and *Tbp* mRNA levels, and at E17.5 of (D) *Nr3c1*
27 mRNA, (E) *Fkbp5* mRNA and (F) *Kcnj12* mRNA, all relative to the mean of *18s*, *Hprt* and *Tbp*
28 mRNA levels. Data, in arbitrary units (A.U.), are means ± SEM and were analysed by 2-way
29 ANOVA with post-hoc Tukey tests (###p<0.001, ##p<0.01, #p<0.05 *vs* vehicle-treated
30 control mice), n=7-17 fetuses from 6 litters per group.
31
32 **Figure 4: Dexamethasone and its metabolite 6-hydroxydexamethasone are present in**
33 **livers of dexamethasone-treated fetuses at E15.5.**
34 Dexamethasone (Dex, stippled bars: 100µg/kg/day) or vehicle (Veh, white bars) was
35 administered to pregnant dams from E12.5 to E15.5, with no further treatment to E17.5.
36 Dexamethasone (A) and 6-hydroxydexamethasone (B) were measured in fetal livers pooled
37 from each litter (approximately 100mg tissue), by mass spectrometry. The detectable range
38 for dexamethasone was 0.01-5ng and for 6-hydroxydexamethasone 0.5-25ng. Values are
39 expressed as ng steroid/g fetal liver. Each data point represents a single pool of fetal livers
40 from one litter. Data are means ± SEM and were analysed by unpaired Student's *t*-test
41 (**p<0.01, ***p<0.001), n=4-6.
42
43 **Figure 5: Suppression of corticosterone levels in fetal liver following dexamethasone**
44 **treatment.**
45 Dexamethasone (Dex, stippled bars: 100µg/kg/day) or vehicle (Veh, white bars) was
46 administered to pregnant dams from E12.5 to E15.5, with no further treatment to E17.5.
47 Steroids were measured in fetal livers pooled from each litter (approximately 100mg tissue),
48 by mass spectrometry. The upper and lower limits of detection of both corticosterone and

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49 11-dehydrocorticosterone (11-DHC) was 0.05-10ng. Levels of (A) corticosterone at E15.5, (B)
50 11-DHC at E15.5, (C) corticosterone at E17.5 and (D) 11-DHC at E17.5. Values are expressed
51 as ng steroid/g fetal liver with each data point representing a single pool of fetal livers from
52 one litter. Data are means ± SEM and were analysed by unpaired Student's *t*-test; **p<0.01,
53 n=4-6.

54

55 SUPPLEMENTARY FIGURE LEGENDS

56

57 Supplementary Figure 1. No association between mean fetal weight per litter and litter size, in
58 vehicle and dexamethasone treated mice.

59 Dexamethasone (Dex: 100µg/kg/day) or vehicle (Veh) was administered from E12.5 to E15.5.
60 Individual data points represent mean fetal weight per litter (g). R2 values were as follows, (A) E15.5
61 vehicle: 0.127, (B) E15.5 dexamethasone: 0.041, (C) E17.5 vehicle: 0.01, (D) E17.5 dexamethasone:
62 0.009. n=10-12 litters.

63

64 Supplementary Figure 2. Levels of mRNA encoding calcium handing proteins are altered at E15.5 by
65 dexamethasone treatment. Dexamethasone (100µg/kg/day, stippled bars) or vehicle (Veh, white
66 bars) was administered to pregnant dams in their drinking water from E12.5 to E15.5. qRT-PCR
67 measurements at (A) E15.5 and (B) E17.5 of cardiac levels of (left to right, respectively) Ryr2 mRNA
68 (encoding RYR2), Atp2a2 mRNA (encoding SERCA2a), Slc8a1 mRNA (encoding NCX1) and Cacna1c
69 mRNA (encoding Cav1.2). E15.5 measurements are relative to the mean of Actb, Hprt and Tbp mRNA
70 levels and E17.5 are relative to the mean of 18s, Hprt and Tbp mRNA levels. Data, in arbitrary units
71 (A.U.), are means ± SEM and were analysed by 2-way ANOVA with post-hoc Tukey tests. At E15.5,
72 ANOVA showed a borderline significant genotype effect (p=0.054) for Ryr2, and significant effects of
73 treatment for Atp2a2 (p<0.05), Slc8a1 (p<0.01) and Cacna1c (p<0.001). At E17.5, ANOVA showed a
74 significant effect of treatment for Cacna1c (p<0.01). Post-hoc tests: ##p<0.01, #p<0.05, n=7-17
75 fetuses from 6 litters per group .

76

77 Supplementary Figure 3. GR protein levels in control fetal hearts at E15.5.

78 Western blotting (representative image in upper panel) was used to measure protein levels in hearts
79 of vehicle (V/Veh) and dexamethasone (D/Dex) treated mice. Graph shows quantification of GR
80 protein levels relative to β-tubulin. Data are means ± SEM and were analysed by unpaired Student's
81 t-test, n= 4 (number of fetuses, from n=2-4 litters per group).

82

83 Supplementary Figure 4. Dexamethasone and its 6-hydroxydexamethasone metabolite are present
84 in livers of dexamethasone-treated dams at E15.5.

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85 Dexamethasone (Dex: 100µg/kg/day) or vehicle (Veh) was administered from E12.5 to E15.5.
86 Steroids were measured in maternal livers by mass spectrometry. The detectable range for (A)
87 dexamethasone was 0.5-50ng, and for (B) 6-hydroxydexamethasone 0.1-5ng. Values are expressed
88 as ng steroid/g liver. Data are means ± SEM and were analysed by unpaired Student's t-test
89 (*p<0.05, **p<0.01). Each data point represents a single dam, n=4-6.

90

91 Supplementary Figure 5. Endogenous glucocorticoid levels in dam liver at E15.5 and E17.5.

92 Dexamethasone (Dex: 100µg/kg/day) or vehicle (Veh) was administered from E12.5 to E15.5. Steroid
93 levels were measured in maternal livers by mass spectrometry: (A) corticosterone at E15.5, (B) 11-
94 dehydrocorticosterone (11-DHC) at E15.5, (C) corticosterone at E17.5 and (D) 11-DHC at E17.5 . For
95 E15.5 samples, the detectable range for corticosterone was 0.025-50ng and 11-DHC, 0.1-50ng. For
96 E17.5 samples, the detectable range for corticosterone was 0.0025-10ng and 11-DHC, 0.0025-50ng.
97 Values are expressed as ng steroid/g liver. Data are means ± SEM and were analysed by unpaired
98 Student's t-test,***p<0.001. Each data point represents a single dam, n=4-6.

99

100 Supplementary Figure 6. SMGRKO fetal HPA axis is downregulated at E15.5 following dex exposure,
101 with no change at E17.5 or in the control 'floxed' group. Corticotropin releasing hormone (Crh)
102 mRNA levels were measured bilaterally in the PVN of the hypothalamus by in situ hybridisation. (A)
103 Representative images of autoradiographs of sections of fetal brain showing hybridisation of Crh
104 mRNA in E15.5 (top panels) and E17.5 (bottom panels) control (left two panels) and SMGRKO (right 2
105 panels) fetal brains. Fetuses were vehicle or dexamethasone exposed, as indicated. (B, C)
106 Densitometric quantification of Crh mRNA at (B) E15.5 and (C) E17.5. Levels of mRNA are expressed
107 in arbitrary units (A.U.) with vehicle treated control set to 1. Data are means ± SEM and were
108 analysed by 2-way ANOVA with post-hoc Tukey tests. (B) There was no significant effect of genotype
109 or dexamethasone treatment individually, but there was a significant interaction (p=0.040). (C) Two-
110 way ANOVA showed a significant effect (p=0.037) effect of genotype. n= 5-13 (number of fetuses,
111 from n=2-5 litters per group).

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TABLE 1: Dexamethasone treatment increased body weight of SMGRKO fetuses at E17.5 but had no effect on fetal body weight or heart weight at E15.5.

Dexamethasone (100µg/kg/day) or vehicle was administered to pregnant dams in drinking water, from E12.5 to E15.5. Fetuses were collected at E15.5 or E17.5. Fetal body and heart weights are in mg with heart weight also expressed relative to body weight. Data are means ± SEM and were analysed using 2-way ANOVA with post-hoc Tukey's test; $^{**}p<0.01$ *vs* untreated SMGRKO, †$p<0.05$ *vs* dexamethasone-treated controls. Body weight: n= 29-52 fetuses from 5-12 litters/treatment). Heart weight: n=11-28 fetuses from 5-6 litters/treatment).

	E15.5			
	Vehicle		**Dexamethasone**	
	Control	**SMGRKO**	**Control**	**SMGRKO**
Body weight (mg)	420.7 ± 20.1	435.6 ± 18.0	459.0 ± 14.6	428.6 ± 14.9
Heart weight (mg)	12.4 ± 1.0	12.8 ± 0.7	13.7 ± 0.7	14.0 ± 0.9
Heart weight/ body weight	0.034 ± 0.003	0.035 ± 0.002	0.036 ± 0.003	0.035 ± 0.003
	E17.5			
Body weight (mg)	873.9 ± 14.3	850.8 ± 13.4	855.2 ± 22.0	954.5 ± 36.4**, †
Heart weight (mg)	13.1 ± 0.7	13.2 ± 0.7	13.8 ± 0.5	14.3 ± 0.12
Heart weight/ body weight	0.015 ± 0.001	0.016 ± 0.001	0.017 ± 0.001	0.018 ± 0.002

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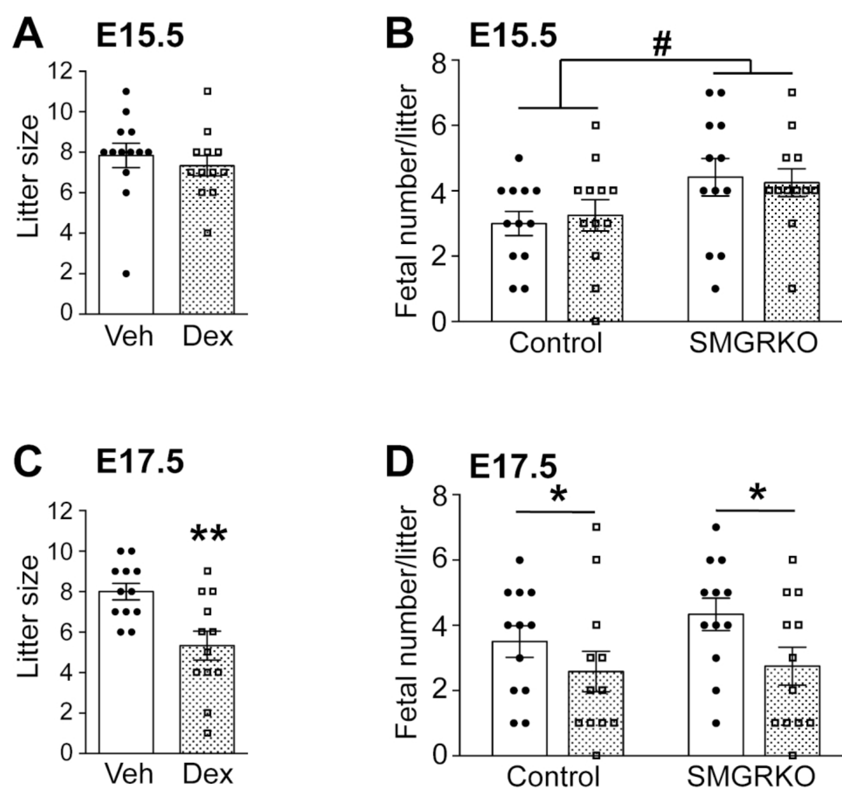

Figure 1

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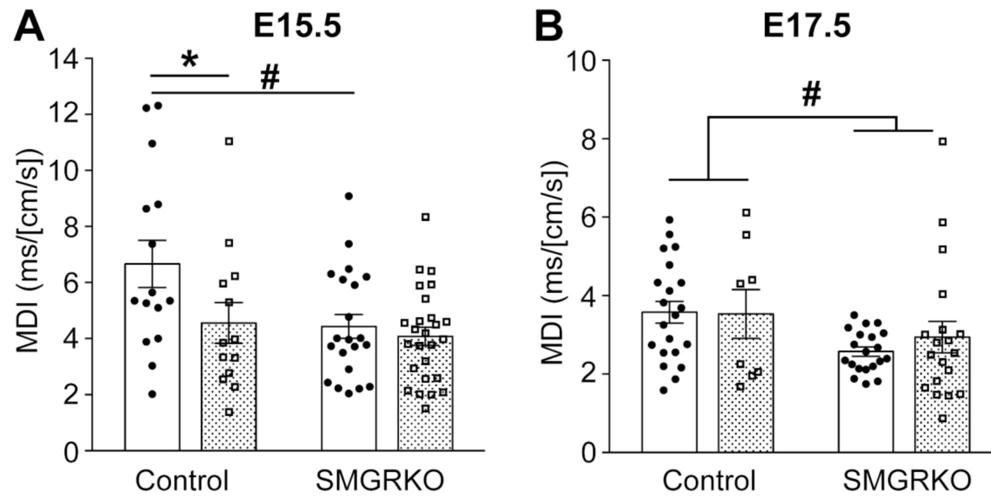

Figure 2

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Figure 3

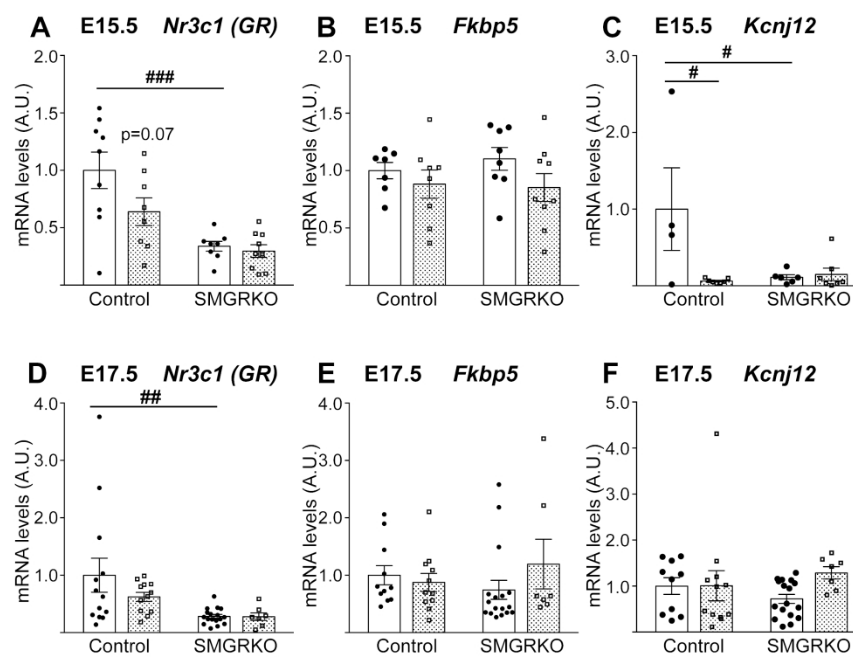

Figure 3

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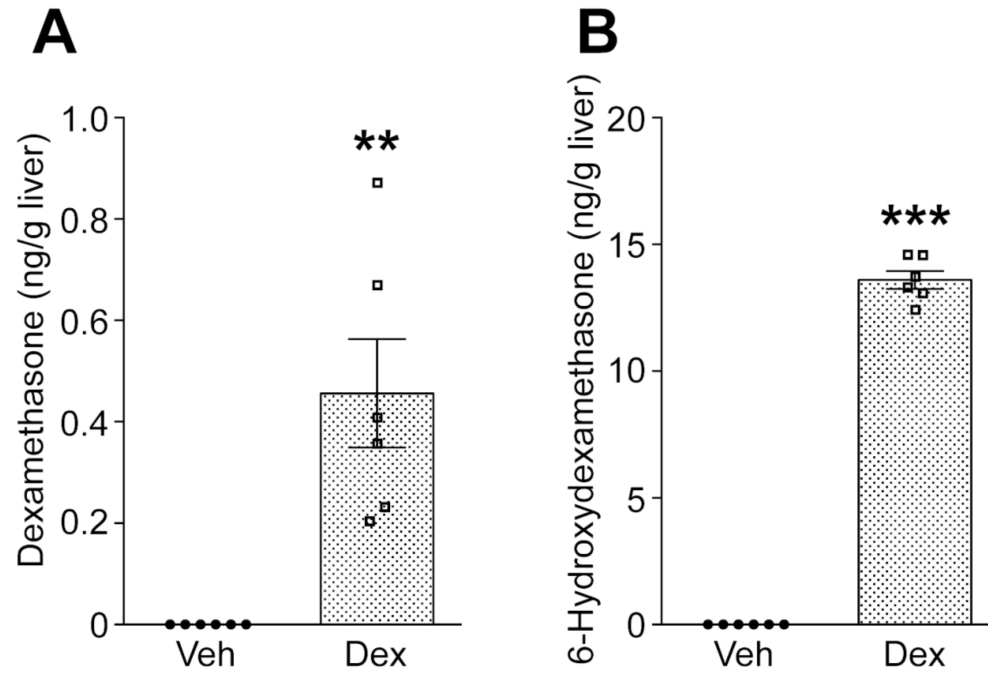

Figure 4

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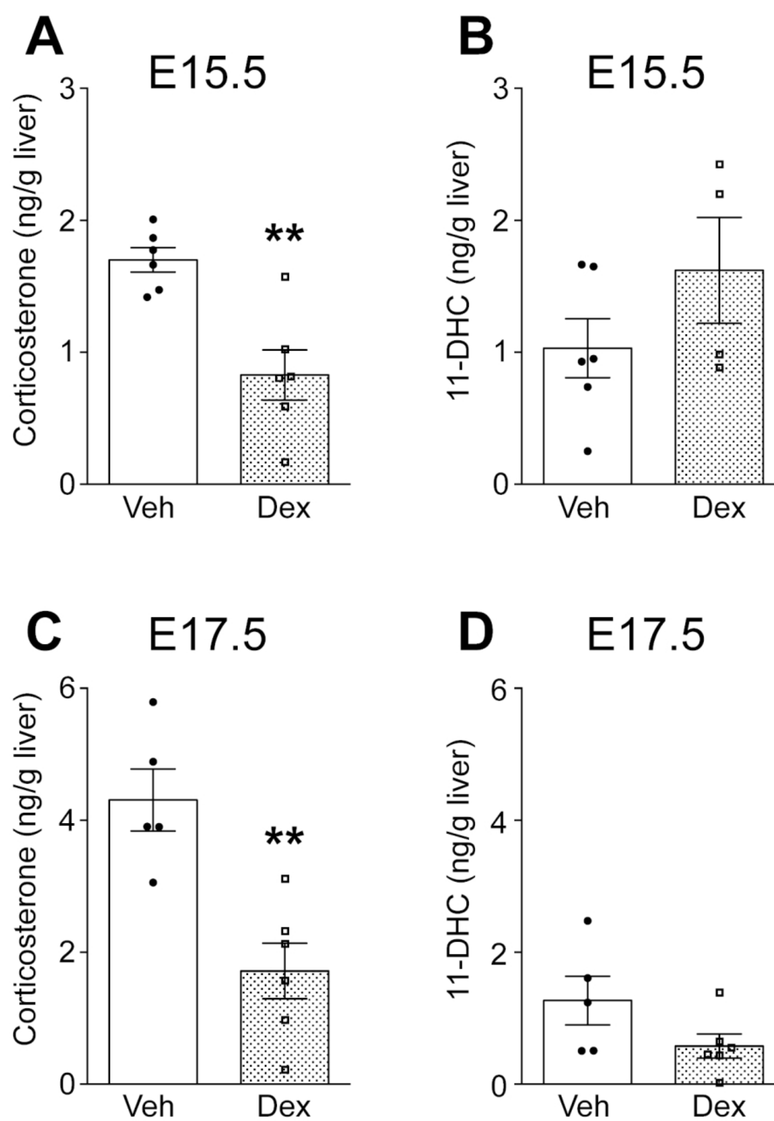

Figure 5

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1 **Supplementary Table 1. Primer sequences and the corresponding probes used for qRT-PCR analysis of mRNA.**
2 Sequences of forward and reverse primers and the Universal Probe Library probe number used in qRT-PCR assays to measure levels of specific
3 mRNAs.
4

mRNA	**Sequence 5'-3'**		**Probe**
	Forward	**Reverse**	
Actb	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA	64
Atp2a2	TCGACCAGTCAATTCTTACAGG	CAGGGACAGGGTCAGTATGC	94
Cacna1c	CCTGCACAAGGGCTCTTTC	AGATGAGGGACACGCTAACC	62
Fkbp5	AAACGAAGGAGCAACGGTAA	TCAAATGTCCTTCCACCACA	97
Hprt	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC	95
Kcnj12	GGCCTAGACCGTATCTTCCTG	TGGCCTCATCAATCTCGTG	9
Nr3c1	CAAAGATTGCAGGTATCCTATGAA	CTTGGCTCTTCAGACCTTCC	91
Ryr2	TTCAACACGCTCACGGAGTA	TGCCAGGCTCTGCTGATT	81
Slc8a1	GTCAGCCTTCAGAGCTGGTC	GACTTCCAACTGCTCCAACC	42
Tbp	GGGAGAATCATGGACCAGAA	GATGGGAATTCCAGGAGTCA	97

5

6

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Supplementary Table 2. Echocardiography parameters measured in E15.5 and E17.5 fetal mice.

Pregnant dams were administered dexamethasone (Dex, 100µg/kg/day) or vehicle (Veh) in their drinking water from E12.5 to E15.5. Fetal cardiac function was assessed at E15.5 or E17.5 using a Visualsonics Vevo 770 High Resolution Ultrasound Scanner using Doppler mode. IVCT: isovolumetric contraction time, IVRT: isovolumetric relaxation time, ET: ejection time, MPI: myocardial performance index, calculated as (IVCT+IVRT)/ET, HR: heart rate measured as beats per minute (bpm), E/A: E/A wave ratio, DT: deceleration time. Data are means ± SEM and were analysed by 2-way ANOVA, with post-hoc Bonferroni tests. P values are stated where p=0.1 to 0.05. Significantly different compared to same treatment and genotype at E15.5, #p<0.05, ###p<0.001, n=7-29 fetuses, from 5-6 litters per group.

E15.5							
	Veh		**Dex**		**ANOVA**		
Parameter	**Control (n=15)**	**SMGRKO (n=24)**	**Control (n=14)**	**SMGRKO (n=28)**	**Treatment**	**Genotype**	**Interaction**
IVCT (ms)	27.36 ± 1.62	27.97 ± 1.11	32.71 ± 2.14	29.09 ± 1.77	p=0.068	NS	NS
IVRT (ms)	40.92 ± 1.48	42.66 ± 1.33	41.43 ± 1.15	40.67 ± 1.09	NS	NS	NS
ET (ms)	109.44 ± 4.44	106.60 ± 2.41	114.08 ± 2.86	106.22 ± 2.65	NS	p=0.090	NS
MPI	0.64 ± 0.04	0.66 ± 0.02	0.65 ± 0.03	0.66 ± 0.03	NS	NS	NS
E/A	0.33 ± 0.03	0.34 ± 0.02	0.36 ± 0.02	0.32 ± 0.02	NS	NS	NS
DT (ms)	56.34 ± 3.68	48.87 ± 2.91	61.99 ± 5.17	53.97 ± 3.52	NS	p=0.054	NS
HR (bpm)	211.1 ± 6.6	216.7 ± 4.3	198.9 ± 6.9	214.4 ± 5.9	NS	p=0.092	NS
E17.5							
	Veh		**Dex**		**ANOVA**		
	Control (n=18)	**SMGRKO (n=23)**	**Control (n=8)**	**SMGRKO (n=20)**	**Treatment**	**Genotype**	**Interaction**
IVCT (ms)	29.58 ± 1.91	31.09 ± 2.03	26.62 ± 1.92	29.43 ± 1.26	NS	NS	NS
IVRT (ms)	35.59 ± 0.98#	33.90 ± 0.85###	36.77 ± 1.06	33.94 ± 1.06###	NS	p<0.05	NS
ET (ms)	99.20 ± 2.06#	104.31 ± 2.20	102.45 ± 2.86	99.02 ± 2.03	NS	NS	p=0.091
MPI	0.68 ± 0.03	0.61 ± 0.02	0.63 ± 0.04	0.61 ± 0.02	NS	NS	NS
E/A	0.47 ± 0.03###	0.46 ± 0.02###	0.45 ± 0.02	0.42 ± 0.02###	NS	NS	NS
DT (ms)	46.35 ± 2.31	44.28 ± 2.37	49.35 ± 3.15	44.85 ± 2.94	NS	NS	NS
HR (bpm)	223.52 ± 6.47	224.16 ± 5.37	215.38 ± 8.87	231.38 ± 6.54	NS	NS	NS

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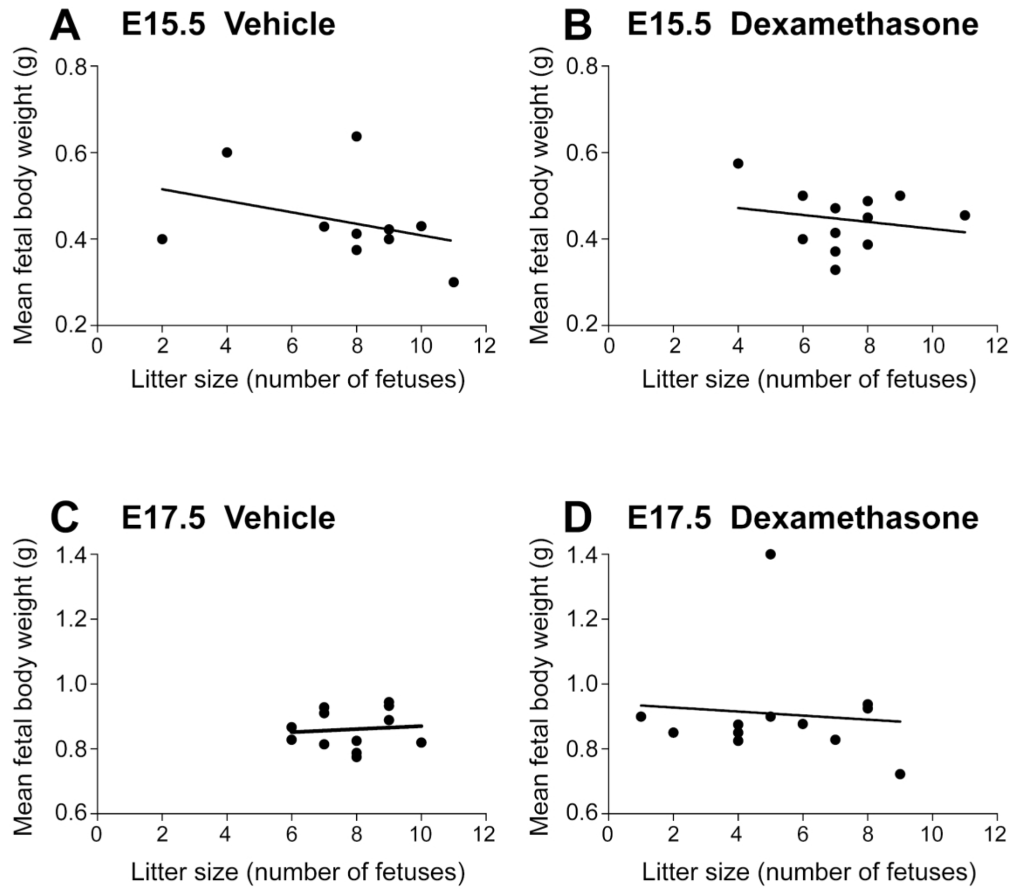
Supplementary Figure 1



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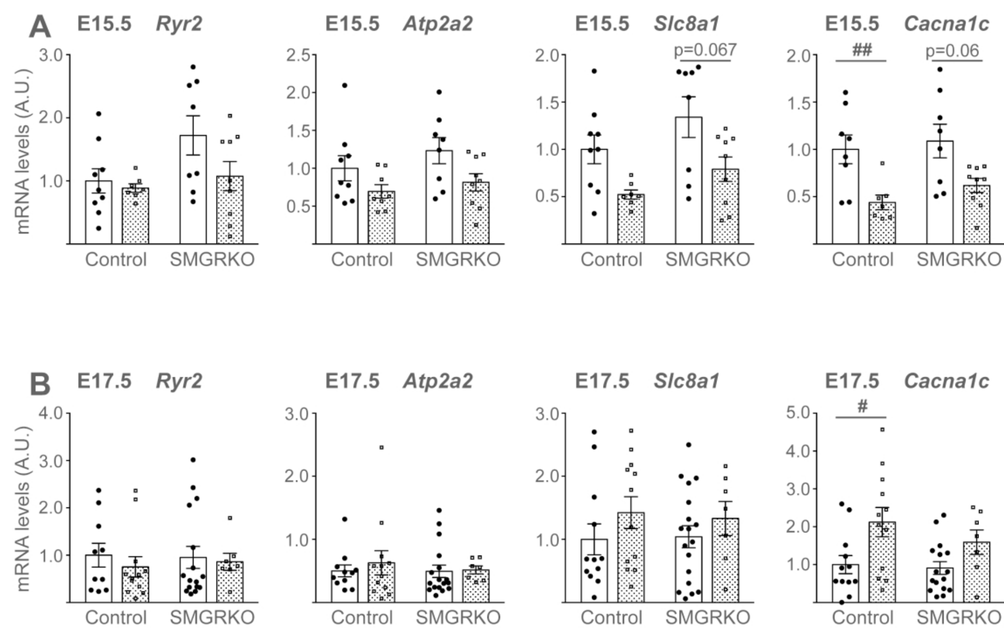
Supplementary Figure 2



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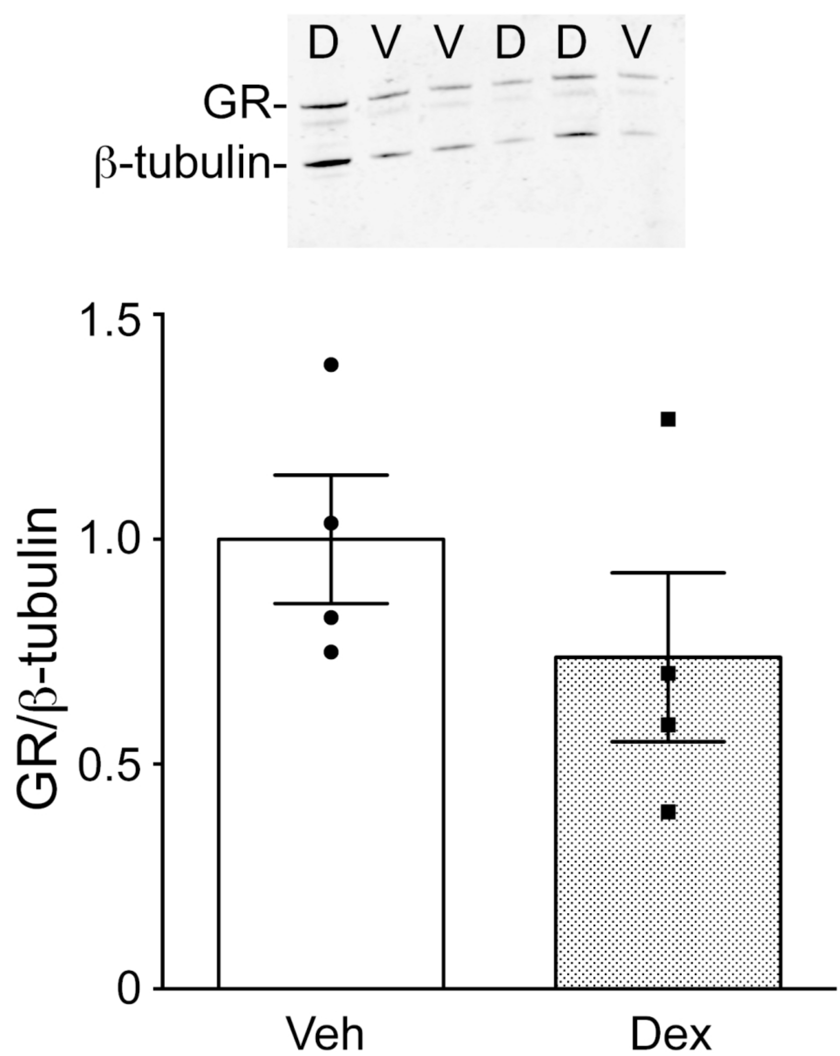
Supplementary Figure 3



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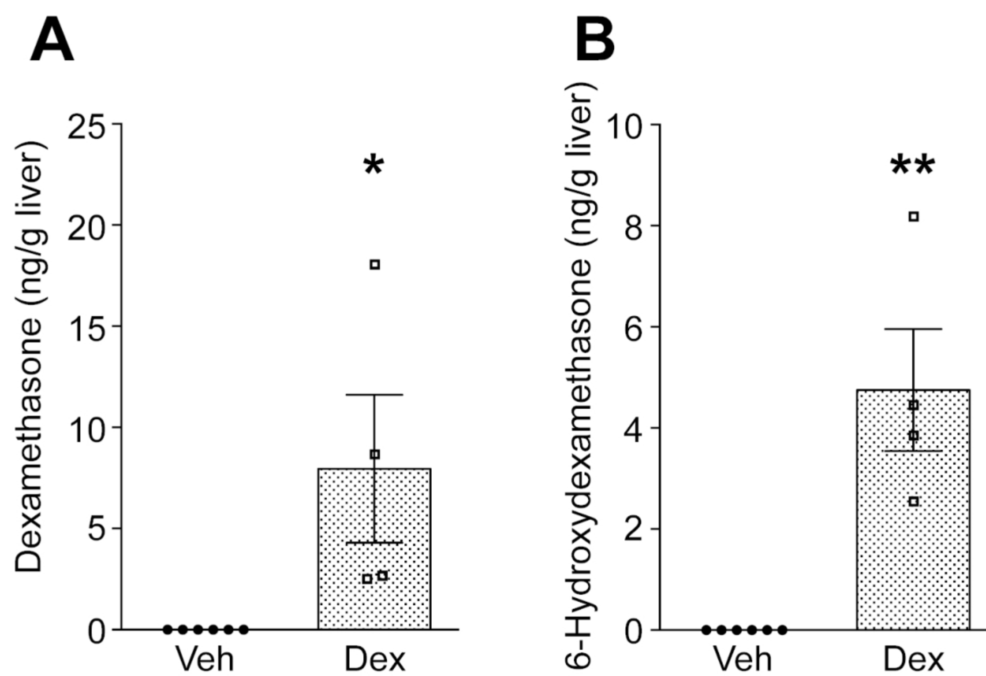
Supplementary Figure 4



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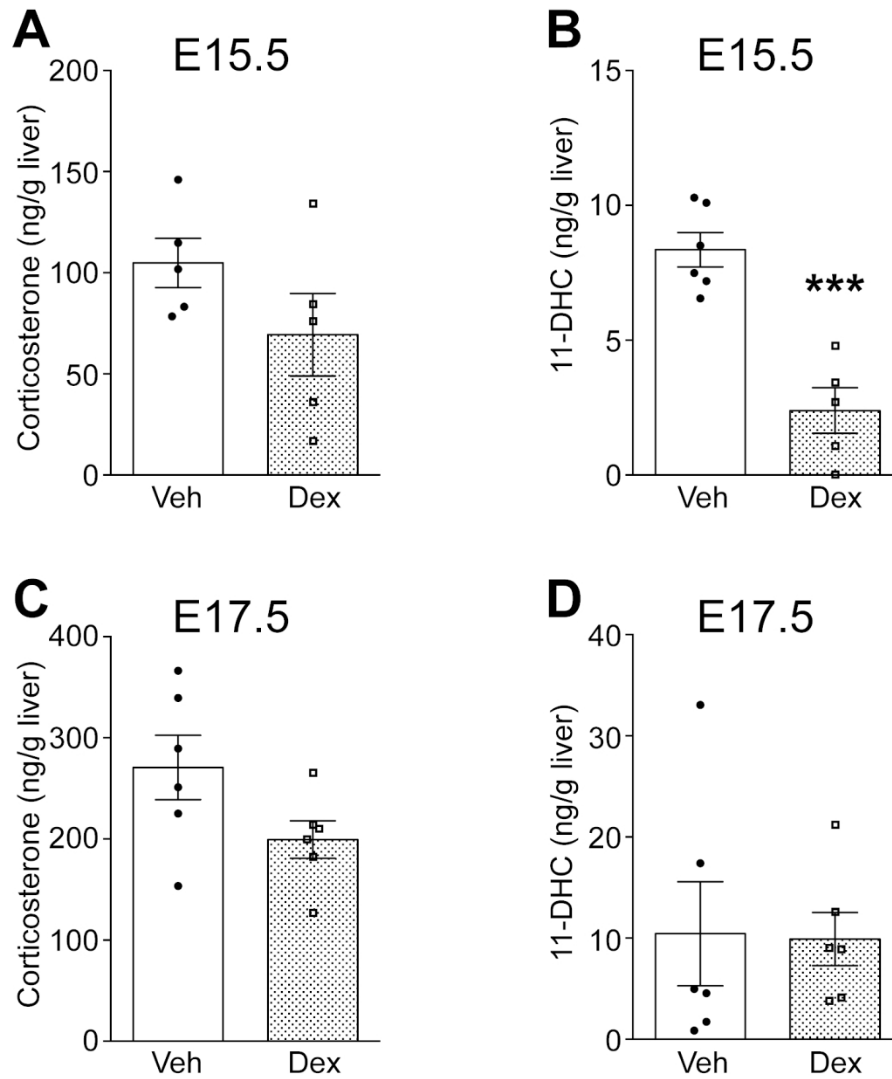
Supplementary Figure 5



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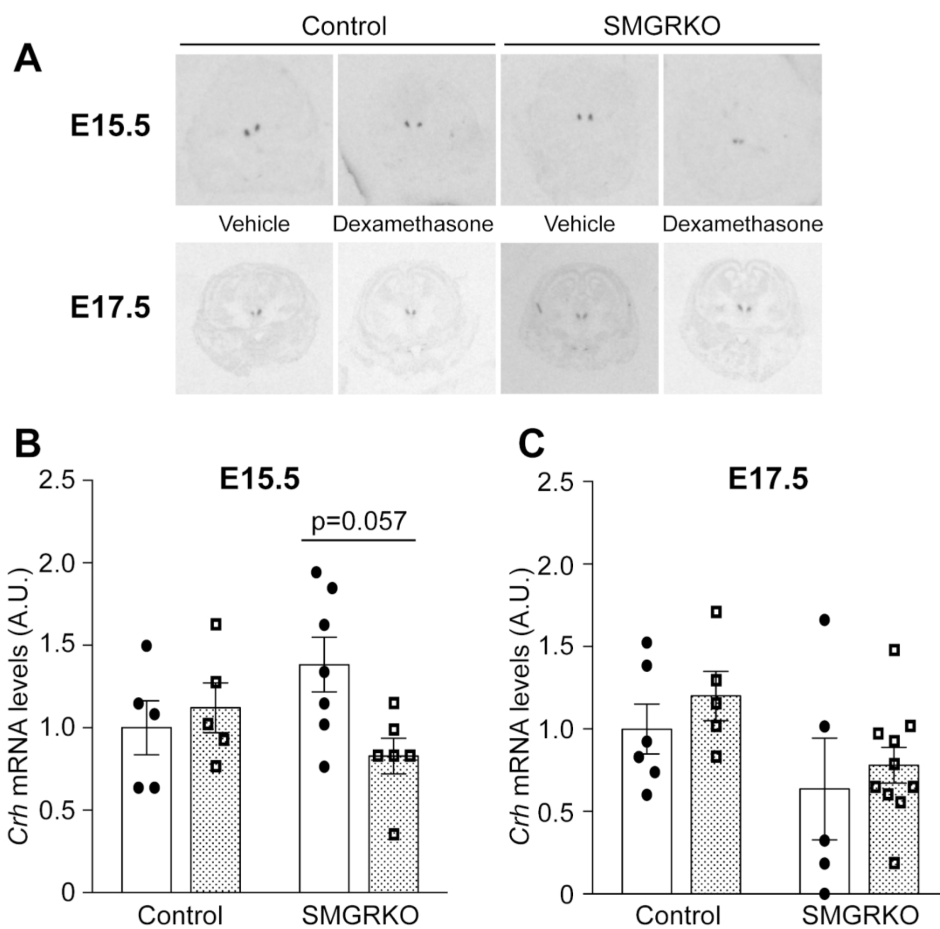
Supplementary Figure 6



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