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Title: Molecular and Behavioral Effects of Infant Maltreatment across Generations in Rhesus Monkeys

Short Title: Intergenerational Infant Maltreatment

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Abstract

Childhood maltreatment is among the most robust risk factors for subsequent psychiatric and medical disorders, and data in humans and rodents suggest that effects of adverse childhood experiences may be transmitted across generations. Recent indications for biological processes underlying this transfer of experiential effects are intriguing; yet, their relevance in primates is inconclusive due to limitations of current studies. In this study, we bridge research in rodent models and humans with a natural non-human primate model on intergenerational effects of childhood maltreatment. Using a unique, well-controlled, randomized cross-fostering design in rhesus monkeys, we test the influence of ancestral maltreatment on molecular, neuroendocrine and behavioral outcomes in offspring, and show that childhood maltreatment results in transmission of information to the subsequent generation independent of behavioral transmission. We further demonstrate differences in the offspring longitudinal DNA methylation profile of the *FKBP5* gene, an important regulator of the hypothalamus-pituitary-adrenal axis in offspring of the maltreatment lineage compared to the control lineage. Finally, we show that differences in *FKBP5* methylation have functional effects on molecular, neuroendocrine and behavioral outcomes in offspring of the maltreatment ancestral line, even if the infants were never exposed to maltreatment nor interacted with their exposed ancestors. Although the molecular mechanism underlying our observations remains unknown, our data point to a potential germline-dependent effect. In summary, our data suggest that history of maltreatment in primates can induce molecular and behavioral changes in a subsequent generation independent of behavioral transmission that may influence risk for mental and physical diseases across generations.

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71 **Introduction**

72

73 Exposure to childhood maltreatment is one the most important risk factors for a variety of
74 psychiatric disorders [1,2]. However, both human studies and animal models suggest that effects
75 of environmental experiences such as maltreatment extend beyond the affected individual,
76 influencing phenotypes in subsequent generations. This sparked a controversial discussion on
77 the prevalence of these phenomena, the underlying mechanisms and their biomedical relevance
78 [3-5]. Strong evidence exists for effects of environmental cues on offspring phenotypes when
79 exposed during *in utero* development [6-8], or for the consequences of parental experience on
80 offspring phenotypes mediated by parental behavior [9]. More recently, experiments in rodents
81 suggested that pre-conceptional exposure to chemicals [10], drugs [11], nutritional abnormalities [12]
82 and stress [13-15] can prompt intergenerational effects or even lead to transgenerational
83 inheritance of specific phenotypes [16]. These events seem independent of *in utero* perturbations
84 or behavioral transmission and point to possible germline-dependent mechanisms. Notably,
85 some of the above studies have even demonstrated intergenerational transmission of
86 phenotypes via *in vitro* fertilization. Although recent indications for similar effects with a focus on
87 psychiatric phenotypes in humans are certainly intriguing, these studies are confounded by
88 genetic, behavioral, nutritional, medical and socio-economic factors [17-26]. In addition,
89 multigenerational studies in humans are extremely lengthy and difficult to control. It thus remains
90 unclear if such mechanisms exist in humans and to what extent they may influence physical and
91 mental health.

92

93 Childhood maltreatment and other forms of early life stress (ELS) may cause alterations
94 in long-term cellular programming of relevant pathways including stress response systems such
95 as the hypothalamus-pituitary-adrenal (HPA) axis [27-30]. Epigenetic processes, often broadly
96 defined in the sense of cellular reprogramming, have been proposed as attractive mechanisms
97 to explain the influence of environmental factors on genome function in general, and the
98 regulation of gene expression in particular [31,32]. However, there is an ongoing debate, particularly
99 in primates, as to *if* and *how* environmental information can be transmitted or even inherited
100 through the gametes across generations, potentially preparing the offspring generation for a
101 similar environment through epigenetic adaptation [16,33]. In this study, we focus on the epigenetic
102 regulation of FK506 binding protein 5 (*FKBP5*), a co-chaperone of the glucocorticoid receptor
103 (GR), and an important regulator of the HPA axis influencing the negative feedback control of
104 cortisol and GR activation across species. Prior genetic and epigenetic studies repeatedly
105 implicated *FKBP5* as a risk factor for psychiatric disorders in response to childhood trauma [34-42].

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106 In fact, we and others have previously shown that childhood maltreatment in humans lead to
107 changes in DNA methylation within the *FKBP5* gene, a dysregulation of the negative feedback
108 mechanism towards the GR, and subsequently a higher risk for stress- and trauma-related
109 disorders [42,43].
110
111 Very similar to humans, non-human primate (NHP) infants exhibit drastic socioemotional
112 and neural development over a protracted postnatal period during which the infant is critically
113 dependent on parental care [44-46]. Infant maltreatment occurs spontaneously in NHP species,
114 both in captivity and in the wild, at rates similar to those seen in humans [47-51]. In rhesus
115 monkeys, infant maltreatment has been operationalized by the co-occurrence of physical abuse
116 and rejection of the infant by the mother during the first 3 to 6 months of life, leading to infant
117 pain and distress and elevations of stress hormones [45,52-55]. Our group has extensively
118 characterized the behavioral, neuroendocrine and neurodevelopmental impact of this
119 naturalistic, translational NHP model of maltreatment. We previously demonstrated maltreatment
120 effects on HPA axis hyperreactivity, telomere length, heightened anxiety and emotional
121 reactivity, impulsive aggression and social deficits during development, effects which are
122 consistent with chronic stress exposure [46,55,56]. Further, infant maltreatment leads to increased
123 amygdala volumes associated with higher emotional reactivity [57] and reduces the integrity of
124 brain white matter tracts that connect cortico-limbic regions important for processing of sensory
125 and emotional stimuli [58]. Strikingly, infant maltreatment in NHPs is a relatively stable maternal
126 trait, repeated with subsequent offspring and transmitted across generations [48,59]. This matches
127 similar observations in humans showing that parents with a history of experiencing maltreatment
128 are more likely to show abusive behavior towards their own children compared to parents
129 without this history [60]. Previous studies revealed that cross fostering of infants from a maltreating
130 line to a control line can often halt this vicious cycle, suggesting that the transmission of
131 maternal maltreatment across generations is partially experiential and independent of genetic
132 effects [59]. However, prior studies using this model did not consider the possible non-genetic
133 transmission of maltreatment-related phenotypes across generations and their relevance for
134 disease risk. Importantly, models of ELS or early rearing experiences in NHP support the
135 hypothesis that effects of nursery rearing, variable foraging demand stress, and variations in
136 normal nurturing behavior, among others, may be transmitted across generations [61-64]. Thus, we
137 sought to use our ELS model with cross-fostering to examine the possibility of transmission of
138 epigenetic risk in a well-controlled design. For this, we randomly assigned newborn macaques at
139 birth from maltreating or competent care lineages to competent care mothers to specifically
140 investigate the effect of pre-conceptional maltreatment in the maternal generation on the

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offspring epigenetic profile and related phenotypes. We carefully generated these animals with high genetic and social rank diversity, by choosing them from different social ranks, matrilines and paternities that were balanced across the experimental groups and sexes [58,59,65].

In summary, using a naturalistic model of ELS in NHPs, this study provides evidence for intergenerational, biological programming of *FKBP5* DNA methylation in response to ancestral maltreatment. Infant maltreatment in mothers, a pre-conceptional adverse experience, leads to a decrease in *FKBP5* DNA methylation, as well as HPA neuroendocrine and behavioral alterations in their offspring in the absence of direct interaction of the biological mother with the progeny (i.e. independent of maternal care). Our data highlight the long-term effects of childhood maltreatment across generations potentially increasing risk for psychiatric disorders.

Results

Study Sample

To investigate the contribution of ancestral maltreatment on the next generation molecular, neuroendocrine and behavioral phenotypes, we focus on infants born to maltreated (MALT) or control (CTRL) biological mothers that were randomly assigned and cross-fostered at birth to control foster mothers with a history of competent, nurturing maternal care (CTRL) following previous protocols [58], thus creating two separate groups: CTRL-to-CTRL and MALT-to-CTRL (**Figure 1**). A summary of descriptive data of the cohort divided by biological mother, foster mother and offspring is provided in **Table 1**. The statistical results are presented as mean ± SD throughout, unless otherwise indicated. Mean age (years) of the biological mothers was 9.93 ± 3.35 (MALT: 9.77 ± 3.86, CRTL: 10.06 ± 3.06, t(18)=0.18, p=0.85); mean age of the foster mothers was 10.43 ± 3.35 (MALT-to-CTRL: 9.69 ± 2.97, CRTL-to-CTRL: 11.04 ± 3.66, t(18)=0.89, p=0.38). Both, biological and foster mothers were experienced multiparas without significant differences with respect to parity (Biological MALT: 4.11 ± 2.76, Biological CTRL: 3.64 ± 2.5, t(18)=-0.40, p=0.69 and Foster MALT-to-CTRL: 3.78 ± 2.22, Foster CTRL-to-CTRL: 4.09 ± 2.81, t(18)=0.27, p=0.78) or gravida (Biological MALT: 4.33 ± 2.78, Biological CTRL: 4.18 ± 2.64, t(18)=0.13, p=0.90 and MALT to CTRL: 4.56 ± 2.56, CTRL to CTRL: 4.82 ± 3.13, t(18)=0.20, p=0.84). Importantly, biological mothers did not differ with respect to social rank distribution (MALT rank [high/middle/low]: 2/4/3, CTRL rank [high/middle/low]: 3/2/6 X^2(2, N=20)=1.68, p=0.43). Similarly, foster mothers did not show differences in social rank (MALT to CTRL rank [high/middle/low]: 3/5/1, CTRL- to-CTRL rank [high/middle/low]: 2/3/6 X^2(2, N=20)=4.11,

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176 p=0.13). Offspring groups did not differ in sex (MALT: M/F=3/6, CTRL: M/F=6/5, X^2(1,
177 N=20)=0.90, p=0.34) or rank movement between biological and foster mothers (MALT:
178 [down/none/up]: 2/2/5, CTRL: [down/none/up]: 4/4/3, X^2(2, N=20)=1.65, p=0.44). Thus, groups
179 were balanced with respect to known relevant confounding variables.
180
181 *Maternal phenotype and FKBP5 DNA methylation in the offspring*
182
183 Similar to evidence shown in humans, maltreatment in monkeys spans generations with females
184 exposed to maltreatment being at higher risk to maltreat their own offspring [48,59,65]. To test the
185 hypothesis that MALT in the biological mothers influenced cellular epigenetic programming in the
186 offspring independent of maternal care, we measured *FKBP5* DNA methylation in intron 7,
187 covering the genomic region investigated before in our human studies on childhood trauma [42]
188 (**Supplemental Figure 1**). Focusing on the ancestral maternal phenotype (MALT vs. CTRL),
189 linear mixed effect models show significant lower methylation in MALT offspring than CTRLs
190 already present at birth at CpGs in and around the second glucocorticoid response element
191 (GRE) of intron 7, which are orthologous to the sites found previously demethylated in the
192 human *FKBP5* gene in childhood trauma studies [26,35,42]. After correction for multiple testing, DNA
193 demethylation at CpG5 remains significant (β=0.28, SE=0.08, p=0.003, $p_{Bonferroni}$=0.018) (**Figure**
194 **2**). Thus, in light of the cross-fostering design, our data provide strong evidence for an
195 intergenerational effect of maltreatment on offspring *FKBP5* methylation mediated though
196 mechanisms independent of behavior, with potential commonalities across primate species. Our
197 data also provide evidence for a decrease in *FKBP5* DNA methylation across the normative
198 development of these macaques between birth and 2 years of life. Across the observation period
199 we detect a mean *FKBP5* DNA methylation difference of close to 25% (MALT: 41.12% ± 14.90
200 vs. CTRL: 65.75% ± 19.08) with marked differences between day 2 and month 6 and a
201 convergence of DNA methylation levels at month 24 (**Figure 2**). This is in line with a
202 developmental increase of cortisol secretion observed in humans and NHPs [66,67].
203
204 *Functional impact of differential methylation within FKBP5 GRE binding sequence*
205
206 We previously provided evidence for a functional role of DNA demethylation in and around GR
207 binding sequences in human *FKBP5* intron 7 [42] with demethylation leading to a stronger GR
208 binding to the intron 7 enhancer element, stronger transcriptional activation of *FKBP5*, thus
209 underlying a dysregulation of the negative feedback on GR function and higher cortisol levels
210 over time. Using a competitive ELISA assay, we now tested how DNA methylation at CpG5,

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211 which is located in the center of the GRE, affects binding of the GR in NHPs. Using 35mer
212 synthetic oligonucleotides with either a methylated or unmethylated CpG5 cytosine, we found
213 that the methylated GRE is less efficient in binding the GR compared to the unmethylated
214 oligonucleotide in the macaque model (Mean absorbance 450/655nm, unmethylated=0.356 ±
215 0.035, methylated=0.476 ± 0.046, t(4)=-3.59, p=0.023). These data suggest that the differential
216 *FKBP5* DNA methylation even at a single CpG site observed *in vivo* in rhesus monkeys could be
217 associated with differential GR binding and GR function at the cellular level.
218
219 *DNA methylation of FKBP5 in biological mothers*
220
221 We next tested the possibility of differential *FKBP5* DNA methylation in the adult biological MALT
222 mothers in comparison to the biological CTRL mothers. However, we did not observe a
223 significant difference in DNA methylation between MALT and CTRL biological mothers (Mean %
224 DNA methylation ± SD, MALT: 90.38 ± 8.29, CTRL: 91.56 ± 9.30, t(15)=0.28, p=0.78)
225 (**Supplemental Figure 2**).
226
227 *FKBP5 methylation and neuroendocrine outcomes in the offspring*
228
229 Prior evidence suggests an important role of *FKBP5* methylation in the regulation of the HPA
230 axis function, in particular in stress-reactive glucocorticoid negative feedback and active stress
231 response [34]. Using hair cortisol measurements at postnatal day 2 and 6 months of age, we
232 aimed to assess *in utero* stress exposure of the newborns [68], and HPA activity during the first 6
233 postnatal months [69], respectively and relate our findings of differential *FKBP5* DNA methylation
234 in the offspring to neuroendocrine regulation.
235 The lack of group differences on day 2 hair cortisol levels (log transformed pg/mg hair)
236 suggest that a potential *in utero* exposure to higher stress levels in MALT newborns is less
237 conceivable and does not explain lower *FKBP5* methylation levels in MALT newborns (MALT:
238 6.36 ± 0.28, CTRL: 6.28 ± 0.2, t(18)=-0.73, p=0.47) (**Supplemental Figure 3A**). No significant
239 correlations were detected, either, between postnatal day 2 *FKBP5* DNA methylation and hair
240 cortisol levels in either the entire sample nor the individual groups, which supports the
241 hypothesis that prenatal *in utero* stress exposure is not the primary cause of reduced *FKBP5*
242 DNA methylation in MALT newborns (MALT: r(7)=-0.18, p=0.70, CTRL: r(5)=0.35, p=0.56,
243 Fisher z-score=-0.63, p=0.53).
244 We next tested the hypothesis that the observed differences in *FKBP5* DNA methylation
245 exert an effect on postnatal HPA axis activity as measured by cortisol accumulation in hair from

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246 birth through month 6. Similar to our observations at day 2, there were no obvious group
247 differences in hair cortisol levels between offspring from MALT and CTRL mothers (MALT: 4.74
248 ± 0.22, CTRL: 4.79 ± 0.24, t(16)=0.50, p=0.63) (**Supplemental Figure 3B**), which was expected
249 given the non-MALT control environment provided by the CTRL foster mothers. However,
250 *FKBP5* DNA methylation at month 6 showed an inverse correlation with month 6 hair cortisol
251 between both groups (MALT r(8)=-0.76, p=0.031, CTRL: r(8)=0.69, p=0.06, Fisher z-score=-2.9,
252 p=0.003) (**Figure 4**). This suggests a functional relationship between *FKBP5* DNA methylation
253 and cortisol levels over time such that lower *FKBP5* DNA methylation in MALT offspring are
254 associated with higher postnatal hair cortisol levels.

255 We corroborated these observations by analyzing associations with blood plasma cortisol
256 levels. Neither day 2 baseline morning plasma cortisol (log transformed mean in ug/dl) (MALT:
257 1.91 ± 0.25, CTRL: 2.06 ± 0.36, t(15)=0.98, p=0.34) nor month 6 (MALT: 2.50 ± 0.17, CTRL:
258 2.37 ± 0.15, t(13)=-1.52, p=0.15) showed significant group differences. When calculating the
259 mean plasma cortisol levels between day 2 and month 6 as a proxy for plasma cortisol levels
260 over the first 6 months of life, we did not observe significant group differences either (MALT:
261 2.10 ± 0.37, CTRL: 2.18 ± 0.20, t(18)=0.65, p=0.53), which is congruent with our hair cortisol
262 data. Correlations between day 2 plasma cortisol levels and day 2 *FKBP5* DNA methylation in
263 MALT and CTRL offspring, were not statistically significant, either (MALT r(6)=-0.29, p=0.96,
264 CTRL: r(4)=-0.2, p=0.8, Fisher z-score=-0.08, p=0.94). However, similar to our hair cortisol data,
265 the cumulative plasma cortisol levels (day 2 to 6 months) significantly correlate with month 6
266 *FKBP5* DNA methylation in MALT offspring, and shows a significant difference between MALT
267 and CTRL (MALT r(8)=-0.86, p=0.006, CTRL: r(10)=0.58, p=0.08, Fisher z-score=-3.34,
268 p<0.001). These data further support the observation that MALT individuals with lower month 6
269 *FKBP5* DNA methylation have higher postnatal basal cortisol levels (i.e. HPA axis activation)
270 over time.

271 In summary, cortisol data in hair and in plasma show no discernible group differences
272 between offspring born to MALT vs. CTRL biological mothers, congruent with the competent
273 maternal care both groups of infants experienced postnatally. However, offspring of biological
274 MALT mothers that show reduced *FKBP5* DNA methylation levels show a significant negative
275 correlation between DNA methylation and cortisol levels at month 6, but not at day 2 after birth,
276 indicating that reduced DNA methylation may lead to increased cortisol levels postnatally. These
277 data also suggest that although the baseline HPA axis activity is similar in MALT and CRTL
278 offspring at birth, it may be differentially primed for responding to a stressful environment in the
279 case of offspring of MALT biological mothers.
280

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281 *FKBP5 methylation and behavioral outcomes in the offspring*
282
283 Hair cortisol levels and basal morning plasma cortisol levels under non-stressful, resting
284 conditions may not entirely reflect the influence of *FKBP5* DNA methylation on the dynamic
285 regulation of the HPA axis. In absence of more direct readouts of the dynamic HPA axis
286 regulation in our cohort, we aimed to investigate behavioral effects under mild stressful
287 conditions with respect to ancestral phenotype and *FKBP5* DNA methylation. A broad body of
288 literature shows robust effects of childhood maltreatment and ELS on a spectrum of behavioral
289 outcomes in human and NHPs [70-75]. We hypothesized that MALT offspring will show deficits in
290 certain domains related to emotional and stress reactivity, compared to CTRLs. Therefore, we
291 studied the infant's behavioral reactivity at 6 months during the Human Intruder Test (HIT), a
292 standard NHP paradigm that assesses emotional regulation in response to increasing
293 environmental challenging conditions, and used to investigate stress and anxiety-related
294 behavioral responses [56,57,76]. Indeed, data from the HIT at 6 months suggests a functional effect
295 of *FKBP5* methylation on behavioral outcomes, particularly on measures of anxiety, locomotion,
296 freezing and vocalization during the 'alone', 'profile', and 'stare' conditions of the HIT [57,76]. We
297 found a significant group difference for locomotion during the 'profile' condition with MALT
298 individuals showing increased locomotion compared to CTRL (log transformed mean %
299 locomotion during 10 min observation period) (MALT: -2.42 ± 0.65, CTRL: -6.82 ± 3.86, $t(17)=-$
300 3.7, $p=0.004$) (**Figure 5A**). Significant negative correlations were detected between *FKBP5* DNA
301 methylation at month 6 and anxiety-like responses during the 'alone' condition ($r(17)=-0.53$,
302 $p=0.03$), and anxiety behaviors and locomotion during the 'profile' condition ($r(17)=-0.53$, $p=0.03$
303 for anxiety and $r(17)=-0.62$, $p=0.008$ for locomotion), indicative of increased overall anxiety-like
304 behaviors and locomotion in individuals with reduced *FKBP5* DNA methylation (**Table 2, Figure**
305 **5B**).
306
307 **Discussion**
308
309 The transmission of environmental information and experiences across generations and
310 their importance for relevant molecular, physiological, behavioral and medical phenotypes
311 remains a critical, yet still controversial question. Although recent studies in humans may
312 suggest inter- and even transgenerational transmission or inheritance of acquired phenotypes,
313 the limitations of current experimental designs and approaches prevent more definitive answers
314 as to what extent the environment can influence phenotypes across generations and what the
315 underlying mechanisms are in primates.

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316

317 In this study, we leveraged a unique, well-controlled randomized cross-fostering design in
318 rhesus monkeys, where newborn macaques from infant maltreating or competent, nurturing care
319 lineages were adopted and raised by competent, nurturing foster mothers. Importantly,
320 maltreatment appears to be a stable and natural phenotype discernible in captivity and in the
321 wild, which is not provoked through experimental conditions and is thus well suited as a
322 translational model for childhood maltreatment in humans. The cross-fostering design enables
323 us to specifically investigate the influence of the ancestral experience of the biological mother on
324 molecular, neuroendocrine, and behavioral outcomes in the offspring. In our studies, offspring
325 were cross-fostered immediately at birth, and animals grew up under competent maternal care
326 conditions, not exposed to maltreatment and without contact with the MALT biological mother,
327 thus preventing behavioral transmission of information. Moreover, the longitudinal design of this
328 study allowed us to investigate molecular phenotypes across developmental trajectories in the
329 offspring from birth through the juvenile, prepubertal period. Of note, the design excluded major
330 confounding factors as both groups were balanced with regard to sex of the offspring and age,
331 rank, parity, and gravity status of both the biological and foster mothers. In addition, all
332 individuals shared a similar environment with regard to housing, nutrition, veterinary care, and
333 exposure to natural conditions at the Yerkes National Primate Research Center (YNPRC) Field
334 Station in Lawrenceville, GA.

335

336 We show that ancestral maltreatment in the biological mother leads to reduced *FKBP5*
337 DNA methylation in offspring at birth and during the infant period, providing evidence for an
338 intergenerational effect of childhood maltreatment on epigenetic programming in this NHP
339 primate model. These results are in line with our observations in humans exposed to sexual and
340 physical maltreatment during childhood [42] suggesting comparable programming of DNA
341 methylation in *FKBP5* in response to childhood maltreatment across species with similar spatial
342 and directional effects. Moreover, studies in rodents exposed to glucocorticoids demonstrated
343 similar results suggesting conserved mechanisms across a wide range of species [77]. Infant
344 macaques born to biological MALT mothers showed lower *FKBP5* DNA methylation levels than
345 individuals born to biological CTRL mothers, a difference that is already present at day 2 after
346 birth and lasting at least until 6 months of age (**Figure 2**). The convergence of MALT and CTRL
347 offspring methylation profiles may reflect a compensatory role of the control postnatal
348 environment in which all offspring monkeys were raised. The CpGs found demethylated in prior
349 human studies [26,35,42,78-80] and in this report are spatially central to a functional enhancer element
350 that harbors glucocorticoid response elements (GREs) in intron 7 of *FKBP5*. These GREs

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351 regulate the dynamic transcriptional response of *FKBP5* and thus HPA axis activity, the stress
352 response and glucocorticoid negative feedback [26,34,35,42,78,79,81,82]. Our findings in macaques are
353 consistent with that literature, showing that DNA methylation in the central CpG5 position of the
354 *FKBP5* intron 7 GRE in the macaque gene influence GR binding *in vitro*, and are subsequently
355 associated with differential FKBP5 regulation of GR feedback and peripheral cortisol levels.
356

357 Although we cannot definitively exclude the influence of underlying genetic variants in
358 these monkeys, several aspects make this assumption less likely. First, the behavioral
359 phenotype of infant maltreatment in these monkeys is genotype independent, cross fostering
360 effectively stops the transmission of this behavior, excluding a strong genetic effect. Second,
361 individuals included in this study came from different genetic backgrounds as part of the overall
362 breeding scheme of the YNPRC. Third, for a fixed genetic effect, we would assume that DNA
363 methylation differences are rather stable over time and across generations, which is not the case
364 in our data. When testing the biological mothers for their *FKBP5* DNA methylation profiles, we
365 did not observe significant differences. This indicates that the differences we detect during the
366 first months of development in the offspring may not be stable across life, which is in line with
367 our observation that methylation differences between groups show no difference at 24 months.
368 This observation is in contrast to findings in humans where differences in *FKBP5* DNA
369 methylation appear to remain stable into adulthood [35,42]. However, human studies typically
370 involve an environment that remains similar throughout life. Individuals exposed to childhood
371 maltreatment often continue to live in stressful environments as reflected by the subsequent
372 exposure to adult trauma and other factors such as low socioeconomic status [83,84]. Data
373 presented here in fact support the notion that a non-stressful postnatal environment (in this case,
374 competent maternal care postnatally) may mitigate the intergenerational transmission of risk in
375 form of *FKBP5* DNA methylation as seen by the end of the study period where DNA methylation
376 differences disappear between offspring of biological MALT and CTRL mothers. This is also in
377 line with previous observations suggesting that cross-fostering offspring from MALT biological
378 mothers to CTRL foster mothers inhibit the transmission of maltreatment across generations [59].
379 This is indicative of a non-genetic intergenerational transmission of this particular behavior. Our
380 data show that there are, however, other long-term molecular, neuroendocrine and behavioral
381 alterations detectable in offspring born to MALT in contrast to CTRL lineages, independent of
382 postnatal maternal care received. Nevertheless, these differences seem not sufficient to induce
383 the MALT phenotype.
384

385 DNA methylation in *FKBP5* has been associated with the function of the HPA axis,

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386 dynamic cortisol release and negative feedback signaling of *FKBP5* towards the glucocorticoid
387 receptor. We previously provided evidence for a negative correlation of *FKBP5* DNA methylation
388 and cortisol levels in line with a dysregulation of the HPA axis in humans exposed to trauma or
389 severe stress [42]. Thus, we hypothesized that regulation of the HPA axis is altered in MALT
390 offspring compared to CTRL. Hair cortisol concentrations are indicative of prolonged cortisol
391 accumulation and provide a retrospective reflection of integrated cortisol secretion over periods
392 of several months in humans and non-human primates [69,85]. In addition, hair cortisol levels in
393 newborn monkeys are reflective of *in utero* stress exposure [68]. We used hair cortisol collected on
394 day 2 after birth to test whether prenatal stress could explain the lower *FKBP5* methylation in
395 offspring from MALT lineages compared to CTRL offspring. However, the lack of differences
396 between groups ruled out this possibility. This may also point to a mechanism of transmission
397 that includes early effects of maltreatment on the germline cell biology; however, we cannot
398 exclude the possibility of *in utero* stress effects independent of our hair cortisol readout. Lower
399 *FKBP5* DNA methylation levels in humans are associated with less effective negative feedback
400 on the HPA axis and higher cortisol levels over time [26,42,86,87]. In line with these observations, a
401 negative correlation of *FKBP5* DNA methylation at month 6 with elevated HPA axis activity as
402 measured by both elevated hair and plasma cortisol levels at 6 months was observed in
403 offspring from MALT biological mothers. These findings support prior evidence of disturbed
404 feedback mechanisms of *FKBP5* on the GR and provide evidence for important functional
405 effects of *FKBP5* DNA methylation differences in the rhesus offspring. It is important to note that
406 we did not detect group differences in hair and plasma cortisol at month 6, which is actually in
407 line with the function of *FKBP5* as a regulator of the *dynamic* feedback after stress exposure.
408 Since MALT and CTRL offspring are raised in control environmental conditions, we would not
409 expect to be able to detect baseline differences due to a lack of major postnatal stressful
410 conditions for both groups. However, to test the dynamic response of MALT and CTRL offspring
411 to a stressful event, we choose the Human Intruder Test (HIT) a mildly stressful task, similar to
412 the Strange Situation Test used in human children [76,88] that assesses the response to changing
413 environmental conditions and induces stress and anxiety-related behaviors. Testing the NHP
414 offspring in the 'alone' condition primarily assesses anxiety-related and vocalization phenotypes
415 whereas the 'profile' and 'stare' conditions evaluate aggressive behaviors, locomotion and
416 freezing. In line with prior evidence suggesting an exaggerated response to the HIT in monkeys
417 with higher cortisol levels [89], we show a negative correlation between *FKBP5* methylation at
418 month 6 and anxiety-like behavior during the 'alone' condition as well as anxiety and locomotion
419 during the 'profile' condition. This suggests increased overall anxiety and locomotion in
420 individuals with reduced *FKBP5* DNA methylation, which is in agreement with the hypotheses

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421 that lower *FKBP5* methylation, would lead to higher cortisol levels during a stressful task.
422
423 Several human studies support a role for *FKBP5* DNA demethylation in response to ELS
424 [35,42,78-80]. Importantly, a study in survivors of the Holocaust suggested additional intergenerational
425 effects of trauma exposure on *FKBP5* DNA methylation in the offspring [26]. The Holocaust-
426 exposed parental generation showed increased DNA methylation whereas the offspring
427 generation showed decreased *FKBP5* DNA methylation levels in and around the orthologous
428 intron 7 GREs [26]. In addition, lower levels of DNA methylation were negatively correlated with
429 cortisol awakening response, which in general is in line with our observations. As mentioned
430 before, human studies investigating such phenomena are incredibly difficult to control and not
431 surprisingly provoke strong excitement and controversy at the same time. Similar to human
432 studies, as yet we are unable to provide mechanistic insight to the phenomena we report in this
433 study with NHPs at the moment. However, we emphasize that the infant maltreatment model in
434 rhesus monkeys used in this study is highly translational, and well-controlled, thus providing
435 cross-species supportive evidence for intergenerational effects of maltreatment in childhood, one
436 of the most critical risk factors for psychiatric disorders. This model is optimally suited for further
437 understanding of mechanisms of intergenerational transmission of information in primates.
438
439 Overall, our data suggest a role for intergenerational programming of epigenetic patterns
440 in response to childhood maltreatment in primates, independent of behavioral transmission. The
441 observed differences in *FKBP5* methylation show functional effects on HPA axis activity and
442 behavioral outcomes. Thus, childhood maltreatment in primates may induce molecular and
443 behavioral changes in a subsequent generation leading to a higher risk for disease across
444 generations.
445
446 **Methods**
447
448 **Subjects and Experimental Groups:** We used a unique and innovative cross-fostering design
449 with random assignment to experimental group at birth. To investigate the contribution of
450 ancestral maltreatment history on the next generation *FKBP5* methylation and related
451 neuroendocrine and behavioral phenotypes, we focused on infants born to maltreated (MALT) or
452 control (CTRL) biological mothers that were randomly assigned and cross-fostered at birth to
453 control foster mothers with a history of nurturing maternal care (CTRL) following previous
454 protocols [58], thus creating two separate groups: CTRL-to-CTRL and MALT-to-CTRL (**Figure 1**).
455 Thus, we are able to ask specifically how maltreatment in the biological mothers influences

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456 offspring outcomes eliminating the possibility of behavioral effects from the biological mother
457 towards the offspring, including variations in maternal care, as offspring grew up in a control
458 environment different from the biological mother. A total of N=20 infant rhesus monkeys
459 (*Macaca mulatta*), 9 males and 11 females, and their biological and foster mothers, served as
460 subjects for the proposed studies, with 11 infants assigned at birth to the CTRL-to-CTRL group
461 (6 males, 5 females), and the remaining 9 infants assigned to the MALT-to-CTRL group (3
462 males, 6 females). (**Table 1, Figure 1**). Biological and behavioral data were collected
463 longitudinally in the offspring from birth to 24 months of age (juvenile, prepubertal period). All
464 subjects lived with their foster mothers and families in large, complex, social groups maintained
465 at Emory University's Yerkes National Primate Research Center (YNPRC) Field Station in
466 Lawrenceville, GA. These social groups consisted of 75-150 adult females, their sub-adult and
467 juvenile offspring and 2-3 adult males, and allowed us to counterbalance our experimental
468 groups for sex, social dominance rank (high, medium and low social status), maternal
469 parity/gravity/age, and to select subjects from different matrilines (i.e. to be genetically
470 unrelated). Animals were housed in 100ftx100ft compounds with access to climate controlled
471 indoor areas. Standard nutrition (Purina Mills Int., Lab Diets, St. Louis, MO) and seasonal fruits
472 and vegetables were provided twice daily with water access *ad libitum*. All procedures were in
473 accordance with the Animal Welfare Act and the US Department of Health and Human Services
474 "Guide for the Care of Laboratory Animals" and approved by the Emory University Institutional
475 Animal Care and Use Committee (IACUC).
476
477 **Behavioral characterization of maternal care:** Maternal care is a stable and consistent trait
478 across generations with abusive behavior transmitted from mothers to daughters, resulting in
479 maternal lineages showing infant maltreatment or competent maternal care, respectively [45,90].
480 We study these lineages in our colony for several years. We selected the CTRL and MALT
481 biological mothers based on their consistent maternal behavior towards previous offspring as
482 described in detail below. In order to characterize their own exposure to maltreatment, we
483 tracked direct experimental observations by our lab and colony records to confirm that the
484 biological MALT mothers were maltreated themselves as infants. This was available for 6 out of
485 9 biological MALT mothers. Based on the consistency of maltreatment across generations, the
486 remaining 3 biological MALT mothers were defined as maltreated based on the consistent
487 maltreating phenotypes of their mothers. The absence of any records of maltreatment towards
488 the CTRL biological mothers defined the CTRL biological mothers who themselves did not have
489 any records of maltreatment towards previous offspring. All foster mothers in this study were
490 selected based on their consistent competent maternal care towards prior offspring, which was

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491 confirmed through direct observations of their maternal behavior towards fostered infants. A
492 detailed description of the behavioral characterization of competent maternal care (CTRL) in
493 contrast to infant MALT is provided in previous publications [45,48,53,55,58,91]. Briefly, focal
494 observations of maternal care are performed over the first 3 months of the infants life (30 min
495 long observation on separate days: 5 days/week during month 1, 2 days per week on month 2
496 and 1 day/week during month 3, for a total of 16 hours. This observation protocol is optimal to
497 document early maternal care in this species, particularly given that physical abuse is the
498 highest during month. Competent maternal care (CTRL) was defined as typical behaviors such
499 as nursing, cradling, grooming ventral contact and protection of the infant. Maltreatment (MALT)
500 was defined as the comorbid occurrence of physical abuse (operationalized as violent behaviors
501 directed towards the infant that cause pain and distress, including dragging, crushing, throwing)
502 and infant rejection (i.e. prevention of nipple/ventral contact and pushing the infant away)
503 resulting in high levels of infant distress [45,52-55].
504
505 **DNA collection:** Genomic DNA for *FKBP5* DNA methylation analysis was obtained from whole
506 blood consistently collected during the morning hours in EDTA tubes (Sarstedt, Newton, NC) at
507 the following time points during development: Day 2, Day 14 (Week 2), Day 30 (Month 1), Day
508 90 (Month 3), Day 180 (Month 6), Day 720 (Month 24). DNA was extracted using standard
509 procedures. We also obtained DNA samples from all biological mothers (N=20).
510
511 ***FKBP5* DNA methylation analysis:** Based on our previous work on human *FKBP5*, we adapted
512 the rhesus *FKBP5* DNA methylation assay from our human assays interrogating DNA
513 methylation in and around functional glucocorticoid response elements (GREs) in intron 7 of
514 *FKBP5* [42]. We interrogated 6 CpG dinucleotides that are orthologous to the human CpGs
515 investigated before. Briefly, at Varionostic GmbH (Ulm, Germany) genomic DNA was bisulfite
516 converted using the EZ-96 DNA Methylation Kit (Zymo, Irvine, CA). Amplicons, 476bp in length
517 were generated from bisulfite converted DNA using primer P1_F 5'-
518 aggaagagagGTTGTTTTTGGAATTT AAGGTAATTG-3' and P1_R 5'-
519 cagtaatacgactcactatagggagaaggctTCTCTTACCTCCA ACACTACTACTAAAA-3'. PCR reactions
520 contained 10x buffer, HotStarTaq, MM, containing 1,5 mmol Mg^{2+}, dNTPs (Qiagen, Hilden,
521 Germany) (5ul), 5 µM Forward primer (0.5ul) 5 µM Reverse primer (0.5ul), Bisulfite DNA (1ul),
522 H_2O (3ul). PCR cycling conditions were [95C-15'00'', 49x (95C-40'', 58C-40'', 72C-40''), 72C-
523 5'00'', 4C]. Amplicons were then analyzed on the Sequenom EpiTYPER platform in accordance
524 with the manufacturers recommendations and included control standards and non-template
525 control (please see **Supplemental Figure 1** for rhesus *FKBP5* intron 7 assay in comparison to

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526 the human intron 7 assay).
527
528 **Enzyme-linked immunosorbent assay (ELISA):** To test the influence of DNA methylation
529 within the GR binding site in intron 7 on the binding capabilities of GR to the putative GRE, we
530 performed a competitive binding enzyme-linked immunosorbent assay using the TransAM GR kit
531 (catalog no. 45496; Active Motif, Carlsbad, CA). To each microwell coated with canonical GRE
532 sequence oligonucleotides, we added dexamethasone-treated HeLa nuclear extract (5 μg;
533 Active Motif) and 40 pmol of one of four double-stranded oligo DNAs (two
534 competing/experimental sequences plus a negative and positive control). All assays were
535 performed in triplicate. Individual oligos and nuclear extract were added to each microwell and
536 incubated for 1 h. Next, the primary antibody (GR 1:1000) and then secondary antibody
537 (horseradish peroxidase (HRP) - conjugated IgG; 1:1000) was added for 1 h each. After several
538 rinses, a colorimetric reaction to horseradish peroxidase was initiated and stopped after
539 7minutes. Absorbance was measured at a wavelength of 450 nm and 655 nm to determine
540 binding efficiencies. Low absorbance indicates a strong binding of the competitive oligo to the
541 GR and thus a reduction of available GR for detection via antibody binding and HRP-based color
542 change. Vice versa, a high absorbance indicates a less efficient binding of the competitive oligo
543 to the GR that hence binds to the consensus GRE sequences immobilized on the plate, leading
544 to a strong calorimetric signal. Antibodies, positive/negative controls and other reagents were
545 provided in the TransAM GR kit. Custom methylated primer sequences: mGRE-fwd 5'-
546 TCCTGTGAAGGGTACAATC/iMe-dC/GTTCAGCTCTGAAAA-3' and mGRE-rev 5'-
547 TTTTCAGAGCTGAA/iMe-dC/GGATTGTACCCTTCACAGGA-3'. Custom unmethylated primer
548 sequences: GRE-fwd 5'-TCCTGTGAAGGGTACAATCCGTTCAGCTCTGAAAA-3' and GRE-rev
549 5'-TTTTCAGAGCTGAACGGATTGTACCCTTCACAGGA-3' (all IDT Technologies).
550
551 **Behavioral Data:** Emotional reactivity of the offspring to varying degrees of threat was
552 examined at 6 months of age during the Human Intruder Test (HIT; [76]). On the testing day, the
553 trained mother-infant pairs were accessed in their home compound and the infant transported to
554 a novel testing room and placed alone in a testing cage with one side made of clear plexiglass to
555 allow video recording following published procedures [57,58]. The HIT is widely used to assess
556 emotional reactivity (including fearful, anxious and aggressive behaviors) to an unfamiliar human
557 in rhesus monkeys under three different and consecutive conditions, 10 min each, that pose an
558 increasing degree of threat: 1) 'Alone' condition (no human intruder), which elicits "calling"
559 vocalizations (coos), locomotion and exploration aimed to escape; 2) next, an unfamiliar human
560 intruder entered the room and stood 3m from the cage presenting his/her profile to the subject

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561 without direct eye contact ('profile' condition), which poses an uncertain threat that results in
562 behavioral inhibition (freezing behavior), stop of vocalizations and vigilance; and 3) direct eye
563 contact of the intruder with the monkey ('stare' condition), which is a threatening behavior for
564 these animals, which typically stop freezing and respond with aggressive and submissive
565 behaviors and threat vocalization towards the intruder. Immediately following the 30 min HIT, the
566 infants were reunited with their mothers and returned to their social groups. All sessions were
567 video recorded and later scored by two trained coders (inter-rater reliability of >90% agreement)
568 using The Observer XT software (v10.5, Noldus, Inc., Netherlands) and published ethograms
569 and procedures [57,58]. For this study, we focused on the analysis of anxiety, freezing, cooing and
570 locomotion behaviors in line with the role of *FKBP5* and the HPA axis in these behaviors.
571
572 **Hair Cortisol:** Hair cortisol data were obtained as described before [55,85]. Briefly, approximately
573 one square inch area of hair was shaved from the nuchal area of the head on postnatal day 2
574 after birth (to measure cortisol accumulated in hair *prenatally*) and at month 6 (to measure
575 cortisol accumulated in hair that grew between birth and 6 months). The hair was weighed,
576 washed with isopropanol, ground to fine powder and extracted with methanol overnight. After
577 methanol evaporation, the residue was re-dissolved in assay buffer and cortisol was determined
578 using the cortisol ELISA kit (Salimetrics, #1-3002; Carlsbad, CA). Intra- and inter-assay
579 coefficients of variation were <10%.
580
581 **Morning Plasma Cortisol:** Procedures for collection of morning basal blood samples have been
582 described before [67,90]. Blood samples were collected at the same ages as hair cortisol (postnatal
583 day 2, and 6 months) in EDTA tubes and immediately placed on ice. Plasma was separated by
584 centrifugation at 1,000g for 10 min at 4°C and then aliquoted and stored at −80°C until assayed.
585 Plasma concentrations of cortisol were assayed by ultra-performance liquid chromatography
586 electrospray ionization (UPLC-ESI) tandem mass spectrometry in duplicates by the YNPRC
587 Biomarkers Core, following published methods [92].
588
589 **Data Analyses:** Demographic and descriptive data on the sample are given in **Table 1**
590 separated for biological MALT and CTRL groups. Groups show a balanced design with respect
591 to major potential confounding variables (e.g. maternal age, parity, social rank, offspring sex).
592 Between group differences for demographic variables in **Table 1** were determined using
593 independent samples t-tests or chi-square tests. A simple linear mixed effects model was used
594 to investigate differences between ancestral groups (MALT and CTRL), in terms of the offspring
595 overall mean *FKBP5* DNA methylation levels across all time points (day 2, week 2, month 1,

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596 month 3, month 6 and month 24); this comparison of means is very similar to a comparison of
597 the area under the curve (AUC approach). *FKBP5* DNA methylation was set as the dependent
598 variable with 6 repeated measures over time; group (MALT, CTRL) was included as a fixed
599 effect and was the effect of primary interest. To account for correlation among the 6 repeated
600 measures and possibly heterogeneous variability over time, the model incorporated a random
601 effect for individual (subject) and separate error variances at the 6 time points. Given our
602 balanced study design with regard to potential confounding factors (biological and foster mother
603 age, rank, parity, gravity; offspring sex), we did not include these variables in the final model,
604 although we first ran preliminary analyses where each of them were separately tested in the
605 model to ensure that they had, indeed, no major influence on the results of the analysis. Linear
606 correlations between variables were performed using Spearman's rank correlation or Pearson's
607 product-moment correlation (two-sided). All analyses were performed in SPSS 24. We used the
608 Shapiro-Wilk Test to confirm normality and log transformed data that deviated from normal
609 distribution. Significance was determined as $p<0.05$. Correction for multiple testing according to
610 Bonferroni was applied for the number of CpGs tested.
611
612
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614
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626
627
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629 MMS performed research; TK, EM, BRH, DG, KJR and MMS analyzed data; and TK and MMS
630 wrote the paper. All authors commented and approved the paper.

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896 **Supplementary Materials**

897

898 Supplementary Figures: S1-S3

899

900 **Figures and Figure Legends**

901

902 **Figure 1**. **Experimental overview**. In order to specifically investigate the effect of infant
903 maltreatment in the ancestral generation, offspring from MALT and CTRL biological mothers
904 were cross fostered after birth to CTRL foster mothers that provided competent care. We
905 followed the offspring from birth to 24 months of life. EDTA blood was obtained at day 2, week 2,
906 month 1, month 3, month 6, and month 24. Hair samples were obtained at day 2 and month 6. A
907 human intruder test was performed at month 6.

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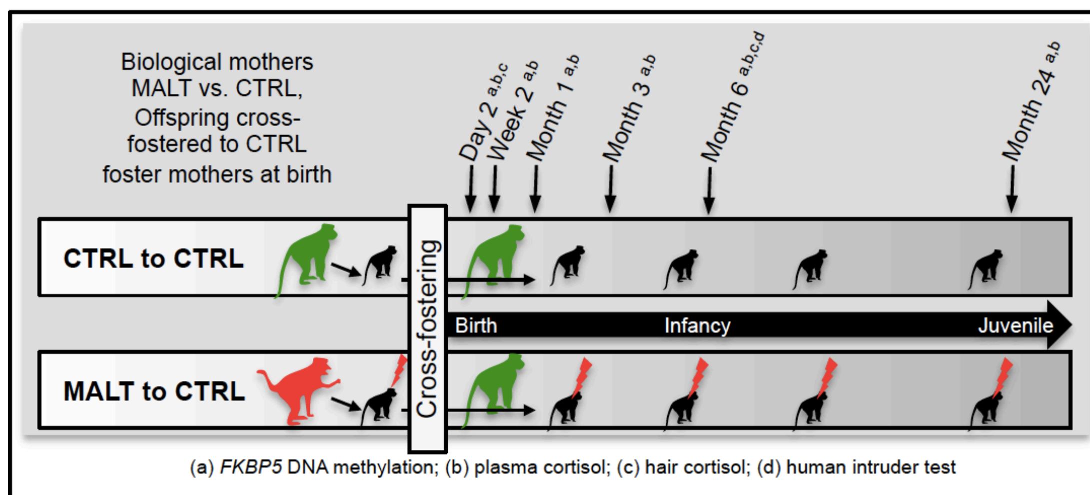

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911 **Figure 2**. **Group-dependent *FKBP5* DNA methylation differences across development**.
912 DNA methylation at previously described CpGs in and around functional glucocorticoid response
913 elements (GREs) was determined using the Sequenom EpiTYPER platform across all 6 time
914 points. MALT offspring (N=9) compared to CTRL offspring (N=12) show lower DNA methylation
915 at CpG5 (β=0.28, SE=0.08, p=0.003, $p_{Bonferroni}$=0.018). DNA methylation differences are
916 pronounced between birth and month 6 and show a convergence at 2 years of age. Error bars
917 indicate SEM.

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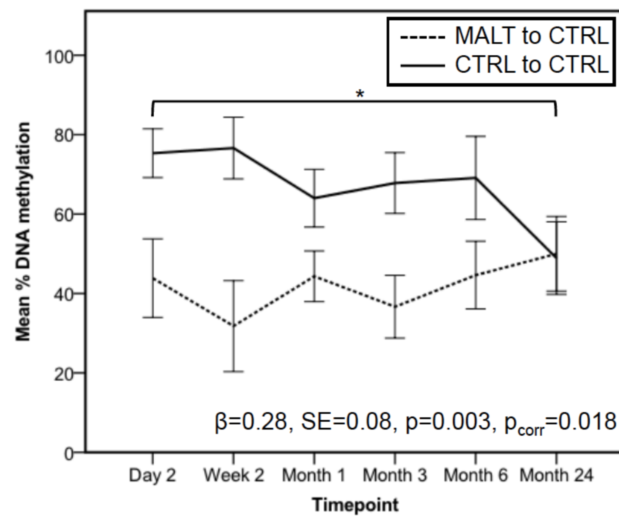

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920 **Figure 3**. **GR binding to methylated and unmethylated GRE sequence**. To test the influence
921 of DNA methylation within the GRE in intron 7 on GR binding, we performed a competitive
922 binding enzyme-linked immunosorbent assay. A low absorbance indicates a strong binding of
923 the competitive oligo to the GR and thus a reduction of available GR for detection via antibody
924 binding and HRP-based color change. Vice versa, a high absorbance indicates a less efficient
925 binding of the competitive oligo to the GR that hence binds to the consensus GRE sequences
926 immobilized on the plate, leading to a strong calorimetric signal. The methylated CpG5 GRE is
927 less efficient in binding the GR compared to the unmethylated oligonucleotide (Mean
928 absorbance 450/655nm, unmethylated=0.356 ± 0.035, methylated=0.476 ± 0.046, $t(4)=-3.59$,
929 $p=0.023$, N=3) indicating that a reduction of DNA methylation within the GRE lead to a stronger
930 GR binding to this enhancer element.

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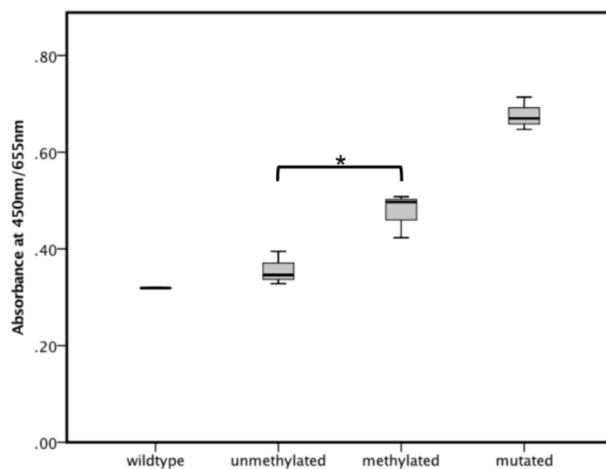

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934 **Figure 4**. **Correlation Month 6 DNAm and Month 6 Hair Cortisol in MALT and CTRL**. A
935 negative correlation between hair cortisol at 6 month and *FKBP5* DNA methylation was
936 observed in MALT offspring (r(8)=-0.76, p=0.031), which is in line with previous reports providing
937 evidence for a reduced *FKBP5* DNA methylation is leading to higher cortisol levels. In contrast,
938 CTRL offspring showed a positive correlation between *FKBP5* DNA methylation and hair cortisol
939 (r(8)=0.69, p=0.06). These data support the notion that *FKBP5* DNA methylation in MALT and
940 CTRL offspring is influencing cortisol levels in opposite directions (Fisher z-score=-2.9, p=0.003)
941 that may lead to functional differences in HPA axis activity under stressful conditions.
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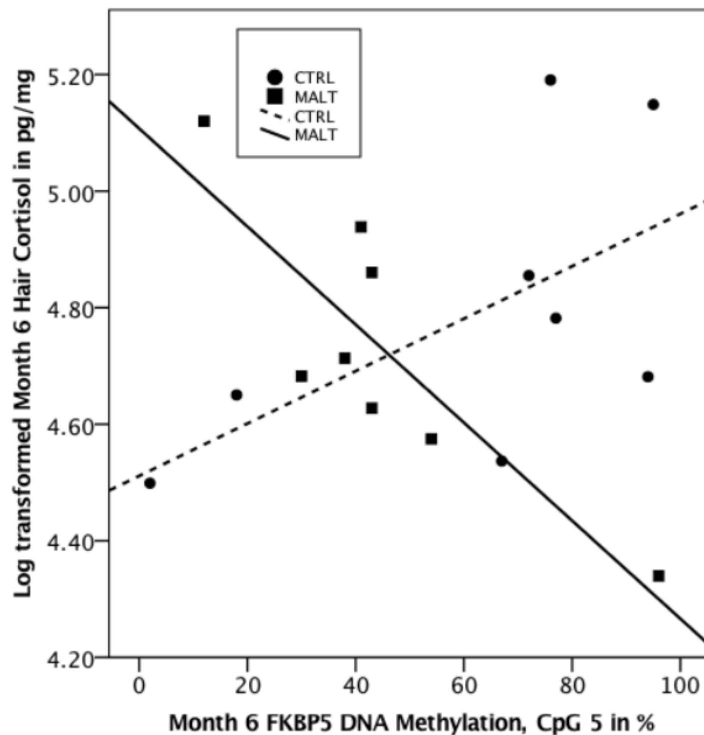

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945 **Figure 5**. **Locomotion during HIT profile condition between MALT and CTRL**. **(A)** Boxplots
946 showing a significant group difference for locomotion during the profile condition with MALT
947 individuals showing increased locomotion compared to CTRL (log transformed mean %
948 locomotion during 10 min observation period) (MALT: -2.42 ± 0.65, CTRL: -6.82 ± 3.86, t(17)=-
949 3.7, p=0.004). **(B)** A negative correlation was detected between *FKBP5* DNA methylation at
950 month 6 and anxiety during the alone condition (r(17)=-0.53, p=0.03), and anxiety and
951 locomotion during the profile condition (r(17)=-0.53, p=0.03 for anxiety and r(17)=-0.62, p=0.008
952 for locomotion), which is indicative of increased overall anxiety and locomotion in individuals with
953 reduced *FKBP5* DNA methylation under stressful conditions.
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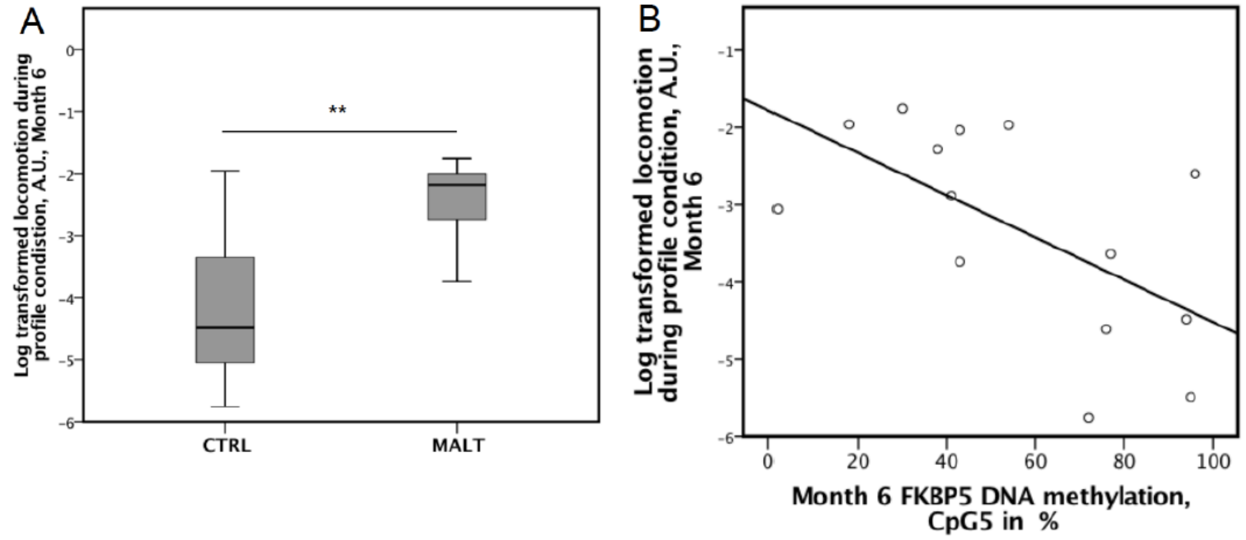

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980 ## Tables and Table Legends

981

982 **Table 1. Demographic data of biological mothers, foster mothers and offspring.**

983 Demographic data for MALT and CTRL biological mothers, CTRL foster mothers, and offspring

984 are shown. Groups were balanced with respect to important confounding variables.

985

Biological Mothers	**MALT**	**Min**	**Max**	**SD**	**CTRL**	**Min**	**Max**	**SD**		**Combined**	**Min**	**Max**	**SD**
Total N	9				11					20			
Mean Age (years)	9.77	5.92	18.27	3.86	10.06	6.94	16.06	3.06	t(18)=0.18, p=0.85	9.93	5.92	18.27	3.35
Mean Gravida (N)	4.33	1	9	2.78	4.18	2	11	2.64	t(18)=-0.13, p=0.9	4.25	1	11	2.63
Mean Parity (N)	4.11	1	9	2.76	3.64	1	10	2.5	t(18)=-0.40, p=0.69	3.85	1	10	2.56
Social Rank (High/Middle/Low)	2/4/3				3/2/6				X^2(2, N=20)=1.68 p=0.43	5/6/9			
Mean DNA methylation CpG5 (%)	90.38[1]	78	100	8.29	91.56[2]	72	100	9.3	t(15)=0.28, p=0.78	91	72	100	8.59
Foster Mothers	**MALT**	**Min**	**Max**	**SD**	**CTRL**	**Min**	**Max**	**SD**		**Combined**	**Min**	**Max**	**SD**
Total N	9				11								
Mean Age (years)	9.69	6	14.02	2.97	11.04	6.94	17.82	3.66	t(18)=0.89, p=0.38	10.43	6	17.82	3.35
Mean Gravida (N)	4.56	2	9	2.56	4.82	2	11	3.13	t(18)=0.20, p=0.84	4.7	2	11	2.81
Mean Parity (N)	3.78	1	7	2.22	4.09	1	10	2.81	t(18)=0.27, p=0.78	3.95	1	10	2.63
Social Rank (High/Middle/Low)	3/5/1				2/3/6				X^2(2, N=20)=4.11 p=0.13	5/8/7			
Offspring	**MALT**				**CTRL**								
Total N	9				11					20			
Sex (M/F)	3/6				6/5				X^2(1, N=20)=0.9 p=0.34	9/11			
Social Rank Movement between Biological and Foster Mother (Down/None/Up)	2/2/5				4/4/3				X^2(2, N=20)=1.65 p=0.44				

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987 Footnotes: [1] N=8 and [2] N=9

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997 **Table 2**. **Spearman's rank correlation of *FKBP5* methylation and behavioral phenotypes**
998 **during the Human Intruder Test in the offspring at month 6**. Table showing the alone (left),
999 profile (middle) and stare (right) conditions and correlations between month 6 *FKBP5*
1000 methylation and functional behavioral readouts. Negative correlations between DNA methylation
1001 and anxiety and locomotor activity indicates that reduces DNA methylation in our cohort is
1002 associated with increased levels of anxiety and locomotion.
1003

		Alone				**Profile**				**Stare**			
		Anx[1]	**Loc**[2]	**Frz**[3]	**Coo**[4]	**Anx**[1]	**Loc**[2]	**Frz**[3]	**Coo**[4]	**Anx**[1]	**Loc**[2]	**Frz**[3]	**Coo**[4]
Month 6 *FKBP5* DNA methy-lation	Spearman's rho	-.529	-0.043	0.243	-0.385	-.532	-.621	0.230	-0.422	-0.151	0.429	0.418	-0.362
	Sig. 2-tailed	**0.029**	0.870	0.348	0.127	**0.028**	**0.008**	0.375	0.091	0.562	0.086	0.095	0.154
	N	17	17	17	17	17	17	17	17	17	17	17	17

1004
1005 Footnotes: [1] Anx=Anxiety; [2] Loc=Locomotion; [3] Frz=Freezing; [4] Coo=Cooing