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**Early life social stress induced changes in depression and anxiety
associated neural pathways which are correlated with impaired maternal
care**

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24

25 **Abstract**

26 Exposures to various types of early life stress can be robust predictors of the
27 development of psychiatric disorders, including depression and anxiety. The
28 objective of the current study was to investigate the roles of the translationally
29 relevant targets of central vasopressin, oxytocin, ghrelin, orexin, glucocorticoid, and
30 the brain-derived neurotrophic factor (BDNF) pathway in an early chronic social
31 stress (ECSS) based rodent model of postpartum depression and anxiety. The
32 present study reports novel changes in gene expression and extracellular signal
33 related kinase (ERK) protein levels in the brains of ECSS exposed rat dams that
34 display previously reported depressed maternal care and increased maternal
35 anxiety. Decreases in oxytocin, orexin, and ERK proteins, increases in ghrelin
36 receptor, glucocorticoid and mineralocorticoid receptor mRNA levels, and
37 bidirectional changes in vasopressin underscore related work on the adverse long-
38 term effects of early life stress on neural activity and plasticity, maternal behavior,
39 responses to stress, and depression and anxiety-related behavior. The differences
40 in gene and protein expression and robust correlations between expression and
41 maternal care and anxiety support increased focus on these targets in animal and
42 clinical studies of the adverse effects of early life stress, especially those focusing
43 on depression and anxiety in mothers and the transgenerational effects of these
44 disorders on offspring.

45 **Keywords: early life stress, depression, anxiety, postpartum depression,**
46 **oxytocin, vasopressin, ghrelin, orexin, mineralocorticoid receptor,**
47 **neuroplasticity.**

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52 **Introduction**

53 Exposures to various types of early life stress can be robust predictors of the
54 development of psychiatric disorders, including depression and anxiety (Eiland and
55 McEwen, 2012; Heim and Binder, 2012; Heim and Nemeroff, 2001; Heim et al.,
56 1997; Johnson and Sarason, 1978; McEwen, 1998; McEwen, 2003). Adverse family
57 social environments are strongly associated with the development of depression
58 (Bouma et al., 2008; Essex et al., 2011; Lizardi et al., 1995) and postnatal exposure
59 to maternal depression has negative effects on offspring mental health (Essex et al.,
60 2011; Goodman et al., 2011). It is postulated that maternal depression exerts its
61 adverse influence through impaired mother-infant bonding (Bureau et al., 2009;
62 Gunnar and Vazquez, 2006; Milan et al., 2009).

63 In rodent dams, chronic social stress (CSS, daily exposure to a novel male
64 intruder) can be used as an ethologically relevant, transgenerational model of the
65 role of stress in the etiology of depression and anxiety in mothers and their
66 offspring (Babb et al., 2014; Carini et al., 2013; Carini and Nephew, 2013;
67 Murgatroyd and Nephew, 2013; Nephew and Bridges, 2011)(see figure 1). Exposure
68 of F0 lactating dams to CSS as a model for postpartum depression and anxiety
69 possesses construct and face validity and depresses maternal care and increases

70 anxiety (Carini et al., 2013; Nephew and Bridges, 2011). For the young F1 offspring
71 of stressed F0 dams, CSS is a robust early chronic social stress (ECSS) which
72 includes exposure to both the depressed maternal care from their F0 mothers and
73 the conflict between the F0 dam and the male intruders. Similar to observations in
74 human mothers exposed to high levels of early life stress (Goodman, 2007), the
75 maternal care displayed by F1 dams towards their F2 offspring is also depressed
76 (Carini and Nephew, 2013; Murgatroyd and Nephew, 2013). Furthermore, the social
77 behavior of both male and female F2 offspring (exposed to depressed maternal care
78 from their F1 mothers) is impaired (Babb et al., 2014). Since maternal depression
79 can often be predicted from an exposure to early life stress, the CSS F1 and F2
80 generations represent relevant models to study the role of ECSS in postpartum
81 depression and anxiety and the adverse effects of these disorders on offspring.
82 Peripheral and central endocrine studies of the CSS model reveal substantial
83 changes in the behaviorally relevant hormones oxytocin (OXT), vasopressin (AVP),
84 prolactin (PRL), estradiol, and corticosterone (Carini and Nephew, 2013; Murgatroyd
85 and Nephew, 2013). In the brain, OXT, AVP, and PRL gene expression are altered in
86 the hypothalamus of ECSS exposed dams (Murgatroyd and Nephew, 2013). OXT,
87 AVP, and PRL are primary mediators of maternal care and have been implicated in
88 the etiology and symptomology of stress related affective disorders (Faron-Górecka
89 et al., 2014; Insel and Young, 2001; Mann and Bridges, 2001; Nephew, 2012; Rilling
90 and Young, 2014; Zamorano et al., 2014). Furthermore, OXT is a key mediator of
91 the reciprocal nature of the mother-infant bond (Carter, 2003; Feldman et al., 2011;
92 Feldman et al., 2007; Henriques et al., 2014; MacKinnon et al., 2014; Mogi et al.,
93 2011). The current study investigated additional neural targets that may be

94 involved in the adverse effects of ECSS on the F1 generation and represent novel
95 preventative or treatment targets.

96 The maladaptive impacts of early life stress on mental health are mediated in
97 part through changes the hypothalamic–pituitary–adrenal (HPA) axis (Gunnar and
98 Vazquez, 2006), although there is growing support for the involvement of multiple
99 interacting brain regions and neuroendocrine factors (Lucassen et al., 2014). There
100 is strong evidence that early life stress has persistent effects on the regulation of
101 the HPA axis through altered gene expression in the brain which involves changes in
102 glucocorticoid receptors (GR) (Liu, 1997; Lupien et al., 2009; McGowan et al., 2009;
103 Suderman et al., 2012) as well as more recent data on mineralocorticoid receptors
104 (MR) (Baes et al., 2014; Juruena et al., 2013; Klok et al., 2011a; Young et al., 2003).
105 It has been suggested that MR receptor activity is increased in patients with
106 depression compared to controls, and a systematic review of the role of GR and MR
107 in early life stress related depression concludes that the effects of stress on the
108 GR/MR ratio may be a key etiological factor in depression, although there are few
109 clinical studies that have investigated this role (Von Werne Baes et al., 2012). Given
110 the previously reported HPA changes in the ECSS exposed F1 dams, it was
111 postulated that changes in both MR and GR may mediate these effects.

112 Recent studies suggest that ghrelin, an orexigenic hormone, may be a
113 primary mediator of the adverse effects of stress on behavior (Ishitobi et al., 2012;
114 Lutter et al., 2008). Although ghrelin is produced in the stomach, it crosses the
115 blood brain barrier (Banks et al., 2002) and ghrelin receptors (GHR) have been
116 found in several brain regions, including the paraventricular nucleus (PVN),
117 amygdala, and ventral tegmental area (VTA) (Alvarez-Crespo et al., 2012; Perello et

118 al., 2012). A Leu72Met polymorphism in the ghrelin gene coding region associates
119 with depression (Nakashima et al., 2008), and elevated ghrelin levels have been
120 found in patients with treatment-resistant depression (Ishitobi et al., 2012),
121 suggesting that ghrelin may be a useful indicator of treatment efficacy. In addition,
122 the orexin system, including the expression of orexin and its receptors (Ox1R and
123 Ox2R) has been implicated in the expression of both maternal care (D'Anna and
124 Gammie, 2006) and depressive behavior and pathophysiology (Arendt et al., 2013;
125 Nollet and Leman, 2013). The clinical and rodent studies indicate that ghrelin and
126 orexin may mediate the depressed maternal care in dams exposed to ECSS.

127 The brain-derived neurotrophic factor (BDNF) pathway, including extracellular
128 signal regulated kinase (ERK) signaling, is another mechanistic target for depression
129 research. In a rat model of infant maltreatment, decreased BDNF levels were found
130 to be programmed through an epigenetic mechanism (Roth et al., 2009). In mice,
131 the inhibition of ERK signaling in hippocampus induces depression-like behavior and
132 blocks the behavioral effects of antidepressants (Duman et al., 2007; Schmidt and
133 Duman, 2007). Adult rodent exposure to exogenous corticosterone affects phospho-
134 ERK1/2 levels in the dentate gyrus, and these effects are sensitive to antidepressant
135 treatment (Gourley et al., 2008). Long-term changes in the BDNF pathway are
136 associated with childhood adversity and adult depression symptoms (Aguilera et al.,
137 2009; Gatt et al., 2009). Mitogen-activated protein kinase phosphatase-1 (MKP-1)
138 expression is increased in the postmortem hippocampus of patients with major
139 depression compared to healthy controls, and increasing MKP-1 activity in rodent
140 models induces depressive behaviors (Duric et al., 2010) and is further regulated by
141 BDNF (Jeanneteau et al., 2010). In the transgenerational CSS model, we propose

142 that ECSS-induced decreases in the BDNF pathway in a nucleus involved in the
143 processing of rewarding stimuli, the nucleus accumbens, may mediate decreased
144 maternal care and increased anxiety.

145 The objective of the current study was to augment the previous investigation
146 of the endocrine and behavioral effects of ECSS in the F1 generation of CSS model
147 of depression and anxiety (Carini and Nephew, 2013) with the addition of
148 neuroendocrine analyses of vasopressin, oxytocin, prolactin, ghrelin, orexin,
149 corticosteroid receptors, and ERK pathways. These targets are both translationally
150 and clinically relevant. This broad, yet targeted, approach was taken because
151 complex behaviors including maternal care are dependent upon multiple interacting
152 brain regions, and ECSS may interact with each of these structures in distinct,
153 meaningful ways. ECSS-induced changes in peripheral estrogen, PRL,
154 corticosterone, maternal behavior, and lactation in the F1 dams in this study have
155 been previously reported (Carini and Nephew, 2013). It is hypothesized that AVP,
156 OXT, PRL, orexin and BDNF related gene expression and/or protein levels will be
157 down regulated in key brain regions in ECSS F1 dams, ghrelin receptors, GR and MR
158 will be up-regulated, and these changes will be associated with ~~impaired/~~
159 maternal behavior.

160 **METHODS**

161 **Animals**

162 Animals in this study were maintained in accordance with the guidelines of
163 the Committee of the Care and Use of Laboratory Animals Resources, National
164 Research Council, and the research protocol was approved by the Tufts Institutional

165 Animal Care and Use Committee. “CSS dams” refers to the adult females exposed to
166 CSS during lactation (F0), and “ECSS dams” refers to the adult female offspring of
167 the CSS dams (F1); the focus of the present study. All the neuroendocrine data and
168 behavioral correlations are from ECSS dams (fig. 1).–

169 **CSS model: creation of F0 dams**

170 Dams (Charles River, Wilmington, MA) mated at Tufts University were
171 subjected to the CSS protocol (previously described by Nephew and Bridges, 2011)
172 consisting of placing a similarly sized (220–300 g) novel male intruder into a
173 lactating female's home cage for 1h from days 2 to 16 of lactation. Control dams
174 were not exposed to the CSS protocol, and were only tested for maternal care and
175 maternal aggression between 0800 and 1200 on days 2, 9, and 16 of lactation (both
176 control and CSS dams were tested for maternal care and maternal aggression on
177 these days). The F1 pups were left in the cage during the intruder presentation and
178 the F1 CSS pups were exposed to depressed maternal care from their F0 mothers
179 and the daily conflict between the mother and the male intruder (Nephew and
180 Bridges, 2011). The rationale for the weekly testing was to avoid introducing
181 potential confounds involved with an excessive amount of dam/pup separations into
182 the model.

183 **ECSS: creation of F1 females**

184 The control and ECSS F1 females of the current study were the offspring of
185 the F0 control and CSS dams; the differences between the treatments of the control
186 and ECSS F1 females were limited to the exposure of the ECSS F1 females to
187 depressed maternal care and daily conflict between their F0 mothers and the male

188 intruders during age 2 to 16 days. The F1 control and ECSS animals were treated
189 identically after the age of 16 days. After weaning all F1 pups on day 23, the female
190 F1 offspring from the twelve F0 control and twelve CSS dams were housed in groups
191 of four until 70 days of age when two from each litter were mated with 6 proven
192 breeder males in groups of 12 (18 F1 females for both the control and ECSS groups).
193 Initial sample sizes for the F1 dams were 14, but 2 control dams were removed
194 from the study due to small litter sizes (5 and 6), resulting in n's of 12 for the
195 control, and 14 for the F1 ECSS group. Total F2 pup number and litter weights were
196 recorded on the day of parturition, and litters were then culled to five females and
197 five males. There were no group differences in the dam weights or number or
198 weights of pups at birth and across lactation (Carini and Nephew, 2013). Total
199 maternal care was defined as the cumulative duration of pup grooming and nursing,
200 and maternal anxiety was defined as the combined duration of self grooming,
201 nesting, and non-maternal locomotion during a 30 minute maternal care
202 observation on days 2, 9, and 16 between 0800 and 1200, the same testing
203 protocol used with F0 maternal care testing. We have previously reported
204 depressed maternal care and/or elevated maternal anxiety in the current ECSS
205 dams throughout lactation, with the most substantial effects on day 2 of lactation
206 and an overall decrease in total maternal care over all three days of testing in the
207 F1 CSS group compared to controls (Carini and Nephew, 2013). The decrease in
208 maternal care was due to significant decreases in the durations of both pup
209 grooming and nursing. While ECSS caused an overall decrease in maternal care
210 throughout lactation (days 2, 9, and 16), the greatest effects on both maternal care
211 and maternal anxiety were during early lactation on day 2 (Carini and Nephew,
212 2013), a critical period for the effects of maternal care on offspring gene expression

213 and development (Champagne et al., 2003; Peña et al., 2013). All F1 dams were
214 euthanized on day 23 of lactation and the brains were extracted and stored at -80C.
215 The final sample sizes at the end of lactation were 12 F1 control dams and 14 ECSS
216 dams.

217

218 **RNA expression analyses**

219 Total RNA and DNA were simultaneously extracted from paraventricular
220 nucleus (PVN), supraoptic nucleus (SON), medial amygdala (MeA), and central
221 amygdala (CeA) brain punches (Bettscheider et al., 2011) and reverse transcription
222 (RT) reactions (Bioline) were performed on 200 ng RNA using random primers to
223 analyze transcript levels. Quantitative PCR (qPCR) was performed on a StepOne
224 Plus (Applied Biosystems) using Sensi Fast SYBR Green (Bioline). Primer sequences
225 and conditions for qPCR reactions are listed in [Table 1](#). Expression levels for OXT,
226 OXT receptor (OXT R), AVP, AVP V1a receptor (AVPR), the long form of the PRL
227 receptor (PRL R), GR (Nr3c1), MR (Nr3c2), Orx1r, Orx2r, Orexin A, and Ghrelin R
228 were normalized against three combined housekeeping genes, β -actin,
229 hypoxanthine phosphoribosyltransferase (Hprt) and glyceraldehyde-3-phosphate
230 dehydrogenase (Gapdh).

231 **Immunoblotting**

232 Protein levels from brain punches of the NAc were analyzed as described
233 previously (Krishnan et al., 2007b). Briefly, samples were homogenized by light
234 sonication in RIPA buffer containing protease and phosphatase inhibitors. Proteins
235 were separated on 4-15% polyacrylamide gradient gels (Criterion System, BioRad),

236 and analyzed by western blotting with the antibodies indicated. Quantification of
237 bands was analyzed by normalizing to corresponding beta-tubulin levels, and
238 phospho-ERK was normalized to total ERK (Image J). Primary antibodies used were
239 against AKT (Cell Signaling 4691; 1:1000), BDNF (Santa Cruz SC-546, 1:500), beta-
240 tubulin (Cell Signaling 2128, 1:1000), ERK1/2 (p44/42 MAPK, Cell Signaling 4695,
241 1:1000), phospho-ERK1/2 (p44/42 MAPK, Cell Signaling 4370, 1:2000), FosB (Santa
242 Cruz SC-48, 1:500).

243 **Statistics**

244 Relative mRNA expression and protein levels were compared with individual
245 ANOVA for each brain region. Where non-significant trends in the ANOVA results
246 were present, these tests were followed with 1-tailed t-tests with Benjamini and
247 Hochberg multiple comparison correction (Benjamini and Hochberg, 1995) if
248 justified by previous studies of the CSS model (OXT and GR). We have previously
249 reported decreased OXT in the MeA (Murgatroyd and Nephew, 2013), and have
250 observed a significant increase in hypothalamic GR expression in the F0 dams which
251 is associated with decreased methylation at the CpG2 promoter region (data
252 submitted for publication). Pearson correlations were used to test for significant
253 gene-behavior associations in restricted data sets (total maternal care and total
254 maternal anxiety on lactation day 2 with the 12 significant differences in gene
255 expression/protein levels (figs. 2-5) in the control and ECSS groups, and both groups
256 combined). All graphical results are presented as mean + SEM, and the level of
257 statistical significance was $p < 0.05$.

258

259 **RESULTS**

260 **Gene and Protein Expression**

261 In the PVN, exposure to ECSS was associated with decreased AVP mRNA
262 expression among F1 dams ($F_{1,25} = 4.1$, $p = 0.05$, Fig. 1A), and increased Ghrelin R
263 ($F_{1,25} = 5.8$, $p < 0.05$, Fig. 1B) and MR ($F_{1,25} = 12.4$, $p < 0.01$, Fig. 1C) mRNA. In addition,
264 the GR/MR mRNA ratio was decreased in the ECSS dams ($F_{1,25} = 8.3$, $p < 0.01$, Fig.
265 1D). In the SON, GR expression was increased in ECSS dams ($F_{1,25} = 3.0$, $p = 0.1$,
266 $t < 0.05$ Fig. 2A), and Orexin A ($F_{1,25} = 4.7$, $p < 0.05$, Fig. 2B), Orx1R ($F_{1,25} = 4.9$, $p < 0.05$,
267 Fig. 2C) and Orx2R ($F_{1,25} = 6.4$, $p < 0.05$ Fig. 2D) were all decreased in stressed dams.
268 In the CeA, ECSS was associated with increased OXTR ($F_{1,25} = 6.1$, $p < 0.05$, Fig. 3A)
269 and AVP ($F_{1,25} = 5.7$, $p < 0.05$, Fig. 3B) mRNA. In the MeA, expression of both OXT
270 ($F_{1,25} = 3.7$, $p = 0.07$, $t = 0.03$, Fig. 3C) and AVP ($F_{1,25} = 5.0$, $p < 0.05$, Fig. 3D) were
271 decreased. Investigation of BDNF and ERK protein levels in the NAc revealed
272 decreased total ERK protein ($F_{1,25} = 13.7$, $p < 0.01$, Fig. 4A) but an elevated
273 phosphorylated ERK/total ERK ratio ($F_{1,25} = 7.7$, $p < 0.01$, Fig. 4B), with a trend for
274 elevated pERK relative to beta-tubulin.

275 **Gene-Behavior Correlations (Table 2)**

276 When correlating gene expression levels with behavioral measures, AVP in
277 the CeA was negatively correlated with maternal anxiety in the ECSS dams; in
278 contrast, AVP in the MeA was positively correlated with maternal anxiety. AVP in the
279 PVN was positively correlated with maternal care and negatively correlated with
280 maternal anxiety in both groups combined. ERK protein levels in the NAc were
281 positively correlated with maternal care and negatively correlated with maternal

282 anxiety in both groups combined. Ghrelin R expression in the PVN was positively
283 correlated with maternal anxiety in both groups combined. GR in the SON was
284 negatively correlated with maternal care in control dams, and negatively correlated
285 with maternal anxiety in stressed dams. MR in the PVN was positively correlated
286 with maternal anxiety in both groups combined. Orexin A and Orx1R in the SON
287 were negatively correlated with maternal care in the control dams, and Orx1R and
288 Orx2R were negatively correlated with maternal anxiety in the ECSS group or both
289 groups combined. Orx2R was also positively correlated with maternal care in the
290 SON.

291 **DISCUSSION**

292 The present study reports novel changes in gene expression and ERK protein
293 levels in the brains of rat dams exposed to chronic ECSS. Substantial changes in
294 vasopressin, oxytocin, orexin, ghrelin, glucocorticoid and mineralocorticoid
295 receptors, and the BDNF pathway underscore related work on the adverse long-term
296 effects of early life stress on neural activity and plasticity, maternal behavior,
297 responses to stress, and depression and anxiety-related behavior. Correlations
298 between gene targets and both groups combined (as found with AVP, ERK, GHR, MR,
299 and Orx2R) indicate that those gene targets mediate the behavioral difference
300 between the two groups. Correlations with only the control or ECSS groups indicate
301 that the changes in those genes mediate behavioral variation within the control or
302 ECSS group, but that the changes are not directly associated with the between
303 group differences or variation in the other group. The gene and protein expression
304 and robust behavioral correlations support increased focus on vasopressin, ghrelin,
305 orexin and changes in both glucocorticoid and mineralocorticoid receptors in both

306 animal and clinical studies of the adverse effects of early life stress, especially those
307 focusing on depression and anxiety in mothers and transgenerational effects on
308 offspring (Apter-Levy et al., 2013; Feldman et al., 2009; Whelan et al., 2015).

309 Several studies have confirmed the importance of AVP in the display of
310 maternal care (Bosch and Neumann, 2008; Bosch and Neumann, 2012; Nephew and
311 Murgatroyd, 2013; Nephew, 2012), and the decrease in AVP in the PVN reported
312 here may mediate the previously documented depressed maternal care and
313 increased anxiety in these F1 dams (Carini and Nephew, 2013), as supported by the
314 positive correlations between maternal care and AVP and negative correlations
315 between maternal anxiety and AVP. Neural AVP promotes ongoing maternal care
316 (Bosch and Neumann, 2008; Nephew and Bridges, 2008b), and the central blockage
317 of AVP V1a receptors at parturition interferes with maternal memory (Nephew and
318 Bridges, 2008a). In CSS exposed F0 dams, exogenous chronic icv AVP treatment
319 ameliorates some of the negative effects of social stress on maternal care (Coverdill
320 et al., 2012). Early life exposure of the F1 dams to depressed maternal care and
321 social conflict may decrease PVN AVP activity through a developmental, possibly
322 epigenetic, mechanism which mediates the impaired maternal care of F1 animals
323 towards their F2 pups (Murgatroyd et al., 2009). It is also possible that this change
324 in hypothalamic AVP may mediate the depressed milk intake in the F1 dams, as
325 both maternal care and lactation are decreased in these dams (Carini and Nephew,
326 2013), similar to comorbid depression and lactational difficulties in humans (Stuebe
327 et al., 2013; Stuebe et al., 2012). In addition to the change in PVN AVP, the
328 expression of this neuropeptide was also altered in the CeA and MeA, and these
329 changes were associated with maternal anxiety on day 2. While the specific

330 functions of the reported changes in amygdalar AVP require further study, it is clear
331 that exposure to ECSS disrupts AVP activity in the brain, and underscores the
332 complex relationship between AVP, maternal behavior, and anxiety (Bosch, 2011;
333 Bosch and Neumann, 2008; Kessler et al., 2011). It is possible that changes in
334 amygdalar AVP were compensatory responses to the increase in maternal anxiety in
335 the ECSS dams, and this hypothesis is supported by the observed changes in
336 expression and correlations between AVP and anxiety. Decreased AVP in the MeA is
337 correlated with maternal anxiety and increased AVP in the CeA is negatively
338 correlated with maternal anxiety.

339 The other significant change in gene expression in the CeA of stressed dams
340 was an increase in OXTR. Similar to the increase in AVP expression, this change
341 may have been part of compensatory mechanism in response to low levels of OXT in
342 the amygdala and/or deficient maternal care. These data add to recent studies
343 implicating disruption in peripheral and central OXT in pathological differences in
344 depressed mothers_(Kim et al., 2014) as well as the growing literature on the role of
345 central OXT in the effects of early social environment on the development of social
346 behavior (Alves et al., 2015) ~~in typical maternal responsiveness and care~~. The lack
347 of significant correlations between the neural changes and maternal behavior in the
348 present study may indicate that the effects of ECSS on maternal care and maternal
349 anxiety may be mediated by a complex array of factors and that future rodent and
350 human studies of oxytocin and maternal care should include additional factors
351 associated with OXT. Oxytocin's beneficial or adverse effects may be mediated
352 through changes in ERK mediated plasticity, AVP, corticosteroid receptors, ghrelin,
353 and/or orexin. Taken together, the hypothalamic and amygdalar AVP and OXT

354 findings support the specific importance of these neuropeptides in the regulation of
355 both maternal care and anxiety in animal models of early life stress-associated
356 disorders.

357 Exposure to ECSS also increases ghrelin R expression in the PVN and this
358 change was correlated with anxiety in both groups combined, similar to reports in
359 male rats (Carlini et al., 2002; Carlini et al., 2004). Chronic icv ghrelin treatment
360 increases depression and anxiety related behaviors in male rats (Hansson et al.,
361 2011), and the PVN appears to be an area particularly sensitive to the anxiogenic
362 effects of ghrelin (Currie et al., 2012), possibly mediated through changes in AVP
363 (Poretti et al., 2015). Serum ghrelin levels in humans are elevated in patients with
364 major depression, and responders to treatments for depression and panic disorder
365 have lower ghrelin levels than non-responders (Ishitobi et al., 2012). On the other
366 hand, ghrelin is reported to mediate resilience to chronic social stress in male mice
367 (Lutter et al., 2008). While more data on developmental changes in peripheral and
368 central ghrelin and ghrelin receptor levels are needed, the current data support the
369 hypothesis that increased ghrelin activity increases maternal anxiety associated
370 behaviors in the his model of ECSS model.

371 Another HPA-related change in the PVN was an increase in PVN MR
372 expression. ECSS increased MR expression in the PVN and lowered the GR/MR ratio,
373 and MR was correlated with maternal anxiety, a maladaptive change in the ECSS
374 group. There is growing evidence of a role for MR activity in the effects of early life
375 stress on HPA development and activity (Baes et al., 2014; Juruena, 2013; Qi et al.,
376 2013; Young et al., 2003). MR haplotypes mediate the cumulative effects of stress
377 on depression symptoms in females (Klok et al., 2011b), and region dependent

378 differences in neural MR gene expression have been reported in humans (Klok et al.,
379 2011a). The present MR data indicate that social stress exposure exerts its long
380 term effects through changes in hypothalamic MR activity, and the correlation
381 between MR in the PVN and anxiety in both groups combined indicates that MR in
382 this region mediates the effect of ECSS on maternal anxiety. Previous studies of the
383 CSS model support the hypothesis that the reported modulation in the GR/MR ratio
384 is disruptive to the HPA axis. Since it has been documented that F1 animals have
385 elevated basal corticosterone at both the adult and dam stages (Carini and Nephew,
386 2013), it is postulated that the increase in PVN MR is in response to increased
387 corticosterone levels and/or reduced GR activity. The present MR data support
388 growing interest in central MR function as a novel treatment target for stress
389 associated psychiatric disorders (Harris et al., 2013; Medina et al., 2013; Otte et al.,
390 2015; Von Werne Baes et al., 2012).

391 The orexin system has been implicated in the pathophysiology of depression
392 (Nollet and Leman, 2013) due to its involvement in the mediation of multiple
393 systems, including arousal, sleep/wake cycles, feeding, stress responses, and
394 reward (Di Sebastiano and Coolen, 2012; Li et al., 2014) and depression associated
395 hypothalamic changes in rodent models (Nocjar et al., 2012). Studies in mice have
396 implicated central orexin activity in the control of maternal care, a robust reward
397 mediated behavior, and maternal aggression (D'Anna and Gammie, 2006). Changes
398 in orexin are also associated with exposure to neonatal maternal deprivation (a
399 robust form of early life stress), and exercise (Feng et al., 2007; James et al., 2014),
400 supporting the hypothesis that orexin may mediate the reported transgenerational
401 effects of ECSS on F1 and F2 offspring. We report hypothalamic changes in orexin

402 activity and depressed maternal care and increased anxiety in dams exposed to
403 ECSS, similar to our previous finding of altered Orx1R expression in the stressed F0
404 mothers of these F1 animals (Murgatroyd et al., 2015)(~~Murgatroyd et al. submitted~~).
405 There was an overall decrease in orexin A activity in the SON, and the associations
406 between Orx2R and maternal care and anxiety in both groups indicates that the
407 ECSS induced changes in behavior are most likely to be mediated by Orx2R, with
408 orexin A and Orx1R being involved in variation in typical maternal care and the
409 individual anxiety response to early life stress. The importance of the orexin
410 receptors in behavioral despair has been previously documented in studies of knock
411 out mice, where an Orx2R knockout displayed increased behavioral despair, with
412 opposite effects in a Orx1R knockout (Scott et al., 2011). Similarly, we report a
413 positive correlation between Orx2R and maternal care, and negative correlation
414 between Orx1R and maternal care. Our data also specifically support recent work
415 reporting decreased hypothalamic orexin A activity in rats exposed to early life
416 maternal separation stress over days 2-14 of lactation (James et al., 2014), another
417 ethologically relevant social stress.

418 Results from the total ERK and pERK analyses reveal that ERK activity was
419 altered in the ECSS dams compared to controls. While the literature on BDNF and
420 depression and anxiety disorders is mixed, the current results add support to the
421 hypothesis that early life stress alters mechanisms of neuronal plasticity. Despite a
422 decrease in total ERK in the NAc, there was a significant increase in pERK relative to
423 tERK and a trend for increased pERK relative to control protein, which is indicative of
424 greater functional ERK activity. Although we did not see significant effects of ECSS
425 on BDNF levels, it is possible that we missed a significant change in BDNF during or

426 shortly after the ECSS exposure which may have had a lasting organizational or
427 epigenetic effects on downstream targets throughout the CNS. The decrease in
428 total ERK may be indicative of attenuated synaptic plasticity and associated
429 behavioral changes (Marsden, 2013). Similar to the current data, total ERK1/2
430 protein levels are decreased in the brains of suicide victims (Dwivedi et al., 2001),
431 and it is postulated that this decrease in the stressed dams is an indicator of
432 depressed maternal care. In terms of pERK, social defeat, another potent social
433 stressor commonly used in males, increases pERK in the VTA (Iniguez et al., 2010)
434 and the NAc, and inhibition of ERK signaling in the NAc blocks the effects of social
435 defeat on depression-like behavior in males (Krishnan et al., 2007a). The relative
436 increase in pERK may mediate the effects of ECSS on maternal care and anxiety.
437 The strength of the associations between ERK levels and maternal care and anxiety
438 could indicate that changes in the expression of other behaviorally significant neural
439 systems (AVP, OXT, orexin, corticosterone receptors) are developmentally mediated
440 by alterations in neuroplasticity in the NAc. Given the importance of plasticity in the
441 maternal brain (Galea et al., 2014; Kim et al., 2010; Kinsley and Lambert, 2008),
442 changes in ERK may be especially relevant to peripartum depression and anxiety.
443 While the current study is limited to the NAc, it is possible that ERK-related gene
444 expression is altered in several other regions, such as the hypothalamus and
445 amygdala.

446 The main limitation of the study is the 20 day interval between the behavioral
447 data and the tissue sampling for gene expression and protein levels. Considering
448 that the exposure to ECSS was during the first two weeks of life of the dams and
449 that the control and ECSS dams were treated identically during the time when they

450 were caring for their own pups, the focus of the present study was on the effects of
451 ECSS on gene expression and behavior that would be expected to be present
452 throughout lactation. While tissue sampling at different time points during lactation
453 would be a valuable component to future studies, prior investigations report that
454 OXT and AVP mRNA and their respective receptor mRNA levels (Nephew et al.,
455 2009) and OXT receptor and AVP V1a receptor binding (Caughey et al., 2011) are
456 relatively consistent across lactation in the PVN, SON, MeA, and CeA of primiparous
457 animals. Furthermore, we focused on targets that are associated with maternal care
458 and/or depression/anxiety both during and not during episodes of impaired maternal
459 care and depression/anxiety, such as plasma oxytocin levels during pregnancy and
460 postpartum depression (Skrundz et al., 2011) and the relationship between early life
461 stress, GR/MR, and depression (Von Werne Baes et al., 2012). Taken together, the
462 design of the CSS model and the focal endocrine targets support the hypothesis
463 that the correlations between behavior during early lactation and gene expression
464 at the end of lactation are relevant for the purpose of identifying factors that may
465 mediate both typical maternal care and the adverse effects of ECSS on maternal
466 care, depression, and anxiety.

467 With growing evidence for stress associated transgenerational mechanisms
468 and mediating roles of early life stress and/or parental behavior in several
469 psychiatric disorders, the current results on long-term alterations in gene
470 expression, protein levels, and robust behavioral associations support continued or
471 additional translational investigation of the roles of vasopressin, oxytocin, orexin,
472 ghrelin, corticosteroid receptors, and neuroplasticity in stress related disorders in
473 mothers and their offspring. Additional investigations of etiological plasticity in the

474 | hypothalamus and amygdala, as well as behavioral gene expression in the nucleus
475 | accumbensNAc, are warranted. A combination of targets that already have clinically
476 | available treatments (often developed for non-psychiatric conditions) and the
477 | preventative potential for future generations (Babb et al., 2015) suggest that
478 | research in these areas may be highly productive in the development of new
479 | treatments and preventative measures.

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869 **Figure 1** Diagram of the Chronic Social Stress paradigm. The current study
870 involved the dams from the F1 generation. Testing on postpartum days 2, 9, and 16

871 included maternal care and maternal aggression. Brain ~~and blood~~ samples from the
872 F1 dams were obtained on postpartum day 23 when the F2 pups were weaned.
873 These samples were analyzed for the expression of oxytocin, oxytocin receptor,
874 vasopression, vasopression V1a receptor, prolactin receptor, glucocorticoid and
875 mineralocorticoid receptors, orexin A, orexin receptors 1 and 2, ghrelin receptor,
876 and protein levels of BDNF, ERK1/2, and phospho-ERK1/2.

877

878 **Figure 2** Mean + SEM relative mRNA expression levels of AVP (A), Ghrelin R (B),
879 MR (C), and GR/MR ratio (D) in the PVN of control (n=12) and ECSS (stress) (n=14)
880 dams. * Indicates a significant effect of CSS, p<0.05

881

882 **Figure 3** Mean + SEM relative mRNA expression levels of GR (A), Orexin (B), Orx1R
883 (C), and Orx2R (D) in the SON of control (n=12) and ECSS (stress) (n=14) dams. *
884 Indicates a significant effect of CSS, p<0.05

885

886 **Figure 4** Mean + SEM relative mRNA expression levels of OXTR (A) and AVP (B) in
887 the CeA and of OXT (C), and AVP (D) in the MeA of control (n=12) and ECSS (stress)
888 (n=14) dams. * Indicates a significant effect of CSS, p<0.05

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890 **Figure 5** Mean + SEM relative protein levels (normalized to beta-tubulin) of total
891 ERK (A) and phospho-ERK/total ERK ratio (B) in the NAc of control (n=12) and ECSS
892 (stress) (n=14) dams. * Indicates a significant effect of CSS, p<0.05

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