Hypothalamic Vasopressin Neurons Enable
Maternal Thermoregulatory Behaviors

Zahra Adahman[1,2,3], Rumi Ooyama[1,2,3], Dinore B. Gashi[2],

Zeyneb Z. Medik[1,4], Hannah K. Hollosi[1], Biswaranjan Sahoo[1,3],

Nana D. Akowuah[1,2,3], Justin S. Riceberg[5], Ioana Carcea[1,3]

1 – Rutgers, The State University of New Jersey, New Jersey Medical School, Department of Pharmacology, Physiology and Neuroscience, Newark, NJ, USA.

2 – Rutgers, The State University of New Jersey, School of Graduate Studies, Health Sciences Campus, Newark, NJ, USA.

3 – Rutgers, The State University of New Jersey, Brain Health Institute, Piscataway, NJ, USA.

4 – Bezmialem Vakıf University, Department of Medicine, Instanbul, Turkey.

5 – Icahn School of Medicine at Mount Sinai, New York, NY, USA.

Corresponding author: Ioana Carcea, ioana.carcea@rutgers.edu

1 **ABSTRACT**

2 Newborns of many mammalian species are partial poikilotherms and require adult

3 thermoregulatory care for survival. In mice, pup survival in cold and cool ambient temperature

4 depends on the ability of adult caregivers to huddle pups and bring them into a high-quality nest.

5 It is therefore essential that adult mice adjust parental care as a function of changes in ambient

6 temperature. Here, we investigated how mouse maternal care adapts to a range of temperatures,

7 from cold to warm. We show that changes in ambient temperature affect several individual and

8 co-parenting maternal behaviors in both dams and virgin female mice, and modulate activity of

9 vasopressin neurons. Furthermore, we establish that the effects of ambient temperature on both

10 maternal care and the activity of vasopressin neurons depend in part on thermosensation,

11 specifically on the TRPM8 sensor. Using trans-synaptic anterograde tracing and whole-brain

12 activity mapping, we find that vasopressin neurons from the paraventricular hypothalamic nucleus

13 connect synaptically with temperature-responsive brain structures implicated in maternal care.

14 We then show that optogenetic activation of vasopressin projections to the central amygdala, a

15 structure activated by cold ambient temperature, recapitulates the effects of cold on co-parenting

16 behaviors. Our data provide a biological mechanism for maternal thermoregulatory behavior in

17 mice with translational relevance to the reported association between ecosystem temperature

18 fluctuations and variations in human child neglect cases.

INTRODUCTION

Changes in ambient temperature associate with the incidence of child maltreatment in humans, with fewer cases of child neglect in cold and more cases in warm temperature [1]. This link could emerge as a consequence of thermoregulation. Endothermic animals developed complex physiological and behavioral adaptations to regulate body temperature [2-5]. In many species additional behaviors evolved in adults to maintain constant core body temperature of poikilothermic infants [6]. In mice, these thermoregulatory behaviors are an essential part of the maternal care repertoire, and include nest building, retrieving pups into the nest, and huddling with pups in nest [7, 8]. Previous work, primarily in rat dams, has shown that cold ambient temperature stimulates pup interactions and nest building, whereas warm temperature decreases time spent in nest [6, 9, 10]. However, the mechanisms underlying these adaptations remain unknown.

Sensing ambient temperature begins with temperature receptors expressed on sensory fibers or on keratinocytes. The majority of temperature receptors are cation channels in the <u>T</u>ransient <u>R</u>eceptor <u>P</u>otential (TRP) family that are gated by ambient temperature. Several TRP channels respond to changes in temperature within the non-noxious temperature range (16º-38ºC). TRPM8 opens at temperatures below 25ºC and is necessary for cold and cool sensing [11, 12]. However, TRPM8 also plays a role in warm processing, as mice lacking TRPM8 (TRPM8 -/-) have deficits in perception of both cold and warm temperatures [13-15]. Information about temperature travels from peripheral sensory neurons to the central nervous system reaching the thalamo-cortical pathway for temperature perception, and the ponto-hypothalamic circuit for coordinated thermoregulatory physiology and behavior [2, 3, 16, 17]. Temperature responsive neurons in the ponto-hypothalamic pathway project to several structures that are important for maternal behavior, including the paraventricular nucleus of the hypothalamus (PVN) [18, 19]. Prior research on maternal care has highlighted PVN as a key brain structure, mediating nest building, pup retrieval, and time spent in nest [20-22]. Within PVN, several peptides have been shown to play important roles in maternal behavior, primarily oxytocin (OT) and vasopressin (AVP) [8]. Whereas the role of oxytocin neurons in enabling maternal behavior in dams and virgin females has been extensively studied [20, 21, 23-25], the role of AVP+ neurons is less well understood. PVN-AVP+ neurons promote pup retrieval and huddling in nest [26], potentially by synapsing on *Galanin*-expressing neurons in the medial preoptic area (MPO), another major regulator of maternal care [7, 27-30]. AVP has also been linked to nest building behavior [31], and to intruder-directed maternal aggression by acting on the central amygdala (CEA) [32].

52 Here, we characterize how acute (maximum 4 hours) exposure to a range of non-noxious

53 ambient temperatures modulates different maternal behaviors in primiparous and nulliparous

54 female mice. We show that thermoreceptors implicated in self-directed thermoregulation also

55 serve maternal thermoregulatory behaviors directed towards pups. Finally, we find that AVP+

56 neurons in PVN respond to ambient temperature and mediate co-parenting but not individual

57 maternal thermoregulatory behaviors via projections to the CEA. Taken together, our study

58 identifies biological mechanisms by which mouse maternal care adapts to changes in ambient

59 temperature, and provides insights into how ecosystem fluctuations influence maternal care in

60 mammals.

61

62 **RESULTS**

63 **Dams and virgin surrogates exhibit maternal thermoregulatory behaviors**

64 To investigate how maternal behaviors of dams and surrogate virgin females adapt to changes in

65 ambient temperature, we employed a sequence of previously described behavioral tests: 1. Four

66 hours of co-housing between dam, virgin and P1-P5 pups, with access to nest, food and water.

67 2. Pup retrieval testing of individual adults (with two P5-P7 pups). 3. Nest building assay for

68 individual adults in the presence of three pups (P7-P14). All behavioral tests were video recorded

69 for behavioral analysis. The initial co-housing also served as priming for virgins, as they gained

70 experience with caring for pups and became maternal, as we previously described [23]. We tested

71 these behaviors at different non-noxious ambient temperatures: cold (CT, 15-17ºC), room

72 temperature (RT, 20-22ºC), thermoneutral temperature (TN, 29-31ºC) and warm temperature

73 (WT, 36-38ºC). We used 70 dams and 79 female virgins who performed at least one of the

74 behavioral tests only once, at one of the four temperatures considered. For some tests we had to

75 exclude data that was identified as outlier, did not meet criteria, or where data collection was

76 compromised (see **Methods**).

77 In the co-housing task we characterized both individual, and dam-virgin co-parenting

78 behaviors. We quantified the time each animal spends in the nest with pups (**Figure 1A**). We find

79 a significant effect of temperature in dams (**Figure 1Ai,** one-way ANOVA, $F_{(3,25)} = 13.44$, p <

80 0.0001, N = 29) and in virgins (**Figure 1Ai**i, $F_{(3,25)} = 4.36$, p = 0.013, N = 29). Pairwise

81 comparison with the RT condition (habitual temperature in the mouse vivarium) shows that dams

82 spend significantly less time in nest when co-housing is done at WT (Dunnett's multiple

83 comparison test, p = 0.0002), and virgins spend significantly more time in nest during CT (p =

84 0.017). Time in nest for all dams and virgins was inversely correlated with ambient temperature

85 (**Figure 1Aiii**, Pearson correlation, r = -0.56, p < 0.0001). We also find that ambient temperature

86　modulated co-nesting, where dam and virgin spend more time together in nest in CT and less

87　time in WT (**Figure 1Aiv**, F(3,24) = 15.61, p < 0.0001; RT vs CT, p = 0.005; RT vs WT, p = 0.016).

88　　　　We previously described an interaction between dam and virgin during the co-housing

89　procedure which we called shepherding, where dams pursuit the virgin towards the nest with pups

90　(**Figure 1B**, [23]). Here we show that the ratio of dam shepherding (out of all pursuits) is sensitive

91　to changes in ambient temperature (**Figure 1Bi**, one-way ANOVA, F(3,18) = 8.54, p = 0.001, N =

92　22). Furthermore, we show that virgins can also shepherd the dams, and the ratio of virgin

93　shepherding is sensitive to changes in ambient temperature (**Figure 1Bii**, Welch's ANOVA,

94　W(3,8.83) = 11.72, p = 0.002, N = 23). Pairwise comparison shows that WT significantly

95　decreases shepherding behavior in virgins (Dunnett's T3 test, RT vs WT, p = 0.024), indicating

96　that pursuits away from nest predominate at WT. Overall, the ratio of shepherding for dams and

97　virgins is inversely correlated with ambient temperature (**Figure 1Biii,** Spearman correlation, r =

98　-0.44, p = 0.002). Ambient temperature does not have a significant effect on pursuit activity, as

99　the number of total pursuits during co-housing did not change with changes in ambient

100　temperature (**Figure 1Biv,** Kruskal-Wallis test, p = 0.689, N = 29 pairs).

101　　　　We next investigated pup retrieval behavior. Despite a trend for WT to decrease

102　performance, pup retrieval success was not significantly affected by temperature in dams

103　(Fisher's exact test, p=0.115). In virgins, we found a significant effect of temperature on success

104　of pup retrieval behavior, with fewer retrievals in WT (p = 0.017). The latency with which virgins

105　retrieved the first pup linearly correlates with ambient temperature, indicating that the colder the

106　environment the faster virgins retrieve (**Figure 1Ciii**, Spearman correlation, r = 0.39, p = 0.024).

107　To determine if virgins bring pups into the nest to thermoregulate the pups or themselves, we

108　repeated the experiment at CT and WT while providing a 'relief' nest: thermoneutral nest in CT

109　environment, and cool nest in WT environment. We find that at CT, virgins retrieve pups faster in

110　the relief nest compared to the nest of same temperature as the environment (**Figure 1Civ**, paired

111　t-test, t(df) = 2.54 (7), p = 0.038, N = 8 pairs). On the contrary, the presence of the relief nest at

112　WT did not significantly speed up pup retrieval (**Figure 1Civ**, Wilcoxon matched-pairs signed rank

113　test, W = -20, p = 0.195, N = 8 pairs).

114　　　　Lastly, we investigated how changes in ambient temperature affected nest building

115　behavior in the presence of pups (**Figure 1D**). The area of the nest built for pups depended on

116　ambient temperature in both dams (**Figure 1Di**, Kruskal-Wallis test, p = 0.0004, N = 30) and

117　virgins (p < 0.0001, N = 40). Compared to the RT condition, dams built smaller nests at TN (Dunn's

118　test, p = 0.044), and virgins built smaller nests at WT (p = 0.043). The area of the nest built by

119 virgins inversely correlates with ambient temperature (Spearman correlation, r = -0.77, p <

120 0.0001). Similarly, the nest quality depended on ambient temperature, as both dams and virgins

121 built lower score nests at higher temperatures (**Figure S1**). Importantly, temperature does not

122 have a significant effect on nest size or quality in the absence of pups, indicating that the effort to

123 build larger and better nests at cold and cool temperatures is motivated by the presence of pups

124 (**Figure 1Div**, Kruskal-Wallis test, p = 0.227, N = 20).
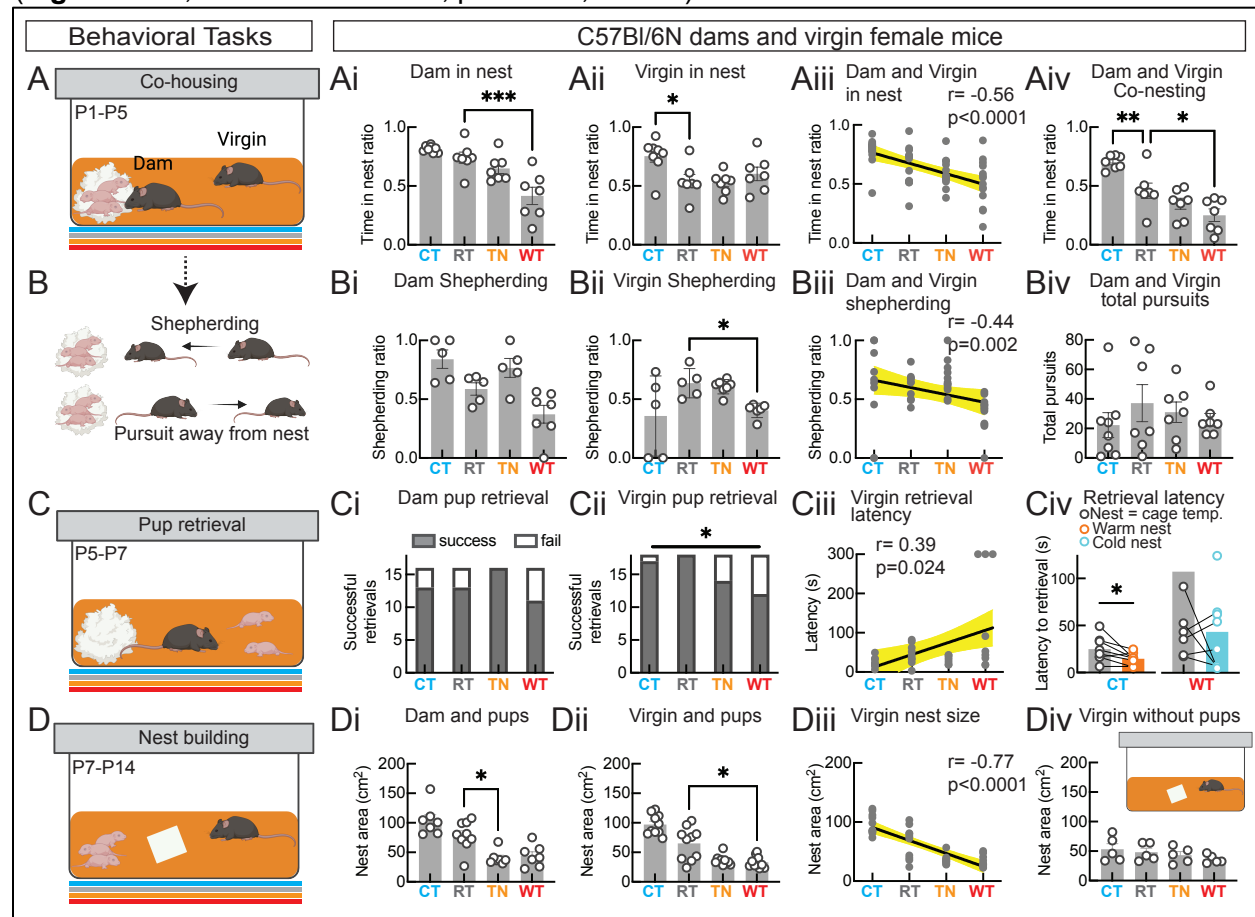
Figure 1: Maternal thermoregulatory behaviors in C57Bl/6N females. **A**. Schematic of the cohousing behavior task. **Ai**. The ratio of time spent in nest by dams. **Aii**. The ratio of time spent in nest by adult virgin females. **Aiii**. Correlation between the ratio of time spent in nest and ambient temperature. **Aiv**. The ratio of time spent co-nesting between dams and virgins at different ambient temperatures. **B**. Depiction of shepherding and pursuits away from nest. **Bi**. The ratio of shepherding by dams. **Bii**. The ratio of shepherding by virgins. **Biii**. Correlation between the ratio of shepherding and ambient temperature. **Biv**. Frequency of all pursuits (toward and away from nest). **C**. Schematic of the pup retrieval behavior task. **Ci.** Dam pup retrieval success. **Cii.** Virgin pup retrieval success. **Ciii.** Correlation of pup retrieval latency in virgins and ambient temperature. **Civ.** Virgin pup retrieval in 'relief' nest. **D**. Schematic of the nest building behavioral assay. **Di**. Area of nests built by dams. **Dii**. Area of nests built by virgins. **Diii**. Correlation of nest area and ambient temperature. **Div.** Area of nests built by virgins in the absence of pups. *p < 0.05, **p < 0.01, ***p < 0.005. Shaded areas in Aiii, Biii, Ciii, and Diii represent 95% confidence intervals.

125   Taken together, the results from the above experiments indicate that female mice adapt
126   pup-directed behaviors to provide maternal thermoregulatory care. The ethological aspects of
127   maternal thermoregulation that we tested do not depend on biological changes associated with
128   gestation, parturition and lactation, as we found similar effects of ambient temperature changes
129   in dams and virgin females. Therefore, to identify biological substrates for maternal
130   thermoregulatory behaviors, we next investigated the contribution of thermosensory mechanisms
131   in female virgin surrogates.

132

133   **TRPM8 -/- mice have impaired maternal thermoregulation**
134   Several mechanistic models could account for the maternal behavior adaptations to ambient
135   temperature. To test if peripheral sensing of ambient temperature plays a role in initiating maternal
136   thermoregulatory behavior, we used mice lacking TRPM8, a cold receptor expressed primarily by
137   sensory fibers [11, 12]. TRPM8 is expressed by both cold-activated and warm-suppressed
138   sensory fibers (**Figure 2A**, [14]) and prior work has shown that TRPM8-/- mice have deficits in
139   both cold and warm sensing [13-15], making this mouse line ideal for investigating the effects of
140   temperature on maternal behavior (**Figure 2A**).

141   In the co-housing experiment, we used TRPM8 -/- virgin females and C57Bl/6N dams and
142   pups. We found that ambient temperature had no significant effect on the time TRPM8 -/- females
143   spent in nest (**Figure 2B**, one-way ANOVA, $F_{(3,24)} = 1.06$, $p = 0.381$, N = 28). Similarly,
144   temperature did not affect the time TRPM8 -/- virgins spent co-nesting with C57Bl/6N dams and
145   pups (**Figure 2Ci,** $F_{(3,24)} = 2.18$, $p = 0.115$, N = 28). Co-nesting behavior differs between
146   C57Bl/6N and TRPM8 -/- virgins (**Figure 2Cii**, two-way ANOVA, effect of genotype, $F_{(3,48)} =$
147   8.40, $p = 0.005$, N = 56). Pairwise comparisons showed that TRPM8 -/- virgins spend less time
148   co-nesting with dams and pups at CT (Benjamini-Krieger-Yekutieli multiple comparison test for
149   C57Bl/6N vs TRPM8 -/- co-nesting, $p = 0.005$ at CT, $p = 0.113$ at RT, $p = 0.255$ at TN, and $p =$
150   0.909 at WT). Shepherding ratio in TRPM8 -/- mice was not affected by temperature (**Figure 2Di**,
151   one-way ANOVA, $F_{(3, 24)} = 1.007$, $p = 0.406$, N = 28), leading to a different pattern compared to
152   C57Bl/6N virgin females (**Figure 2Dii**, two-way ANOVA, genotype X temperature interaction, $p =$
153   0.021, N = 50).

154   Temperature changes still affected nest building behavior in TRPM8 -/- virgins, as they
155   built smaller nests at WT compared to RT (**Figure 2Ei**, $F_{(3,28)} = 10.66$, $p < 0.0001$, N = 32;
156   Dunnett's test for multiple pairwise comparison, RT vs WT, $p = 0.0003$). However, nest building
157   differs between C57Bl/6N and TRPM8 -/- virgin females (**Figure 2Eii**, effect of genotype in two-
158   way ANOVA is $F_{(1,64)} = 25.65$, $p < 0.0001$, N = 72). In pairwise comparisons we find that TRPM8

159   -/- mice build larger nests than C57Bl/6N mice at all temperatures except CT (RT, p = 0.003; TN,

160   p = 0.003; WT, p = 0.016). We found similar effects on nest quality (**Figure S1**).
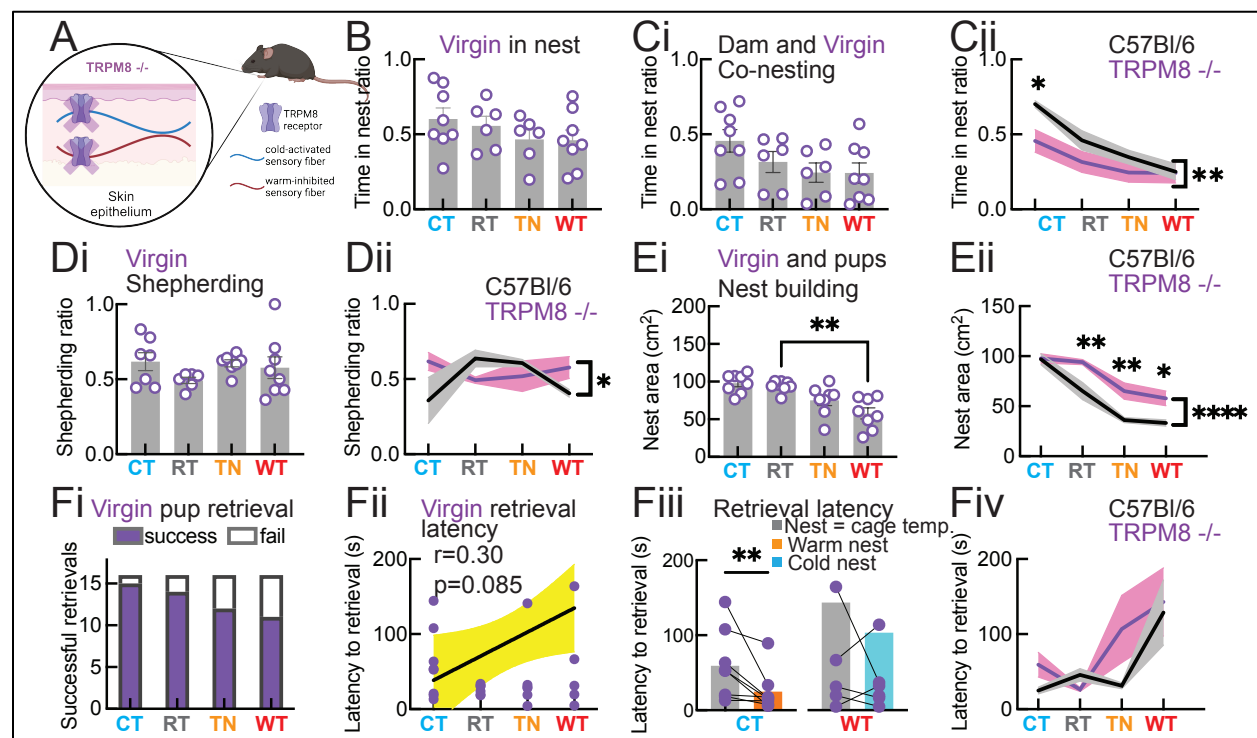
Figure 2: Maternal behavior in TRPM8 -/- virgin females. **A**. Schematic of TRPM8 expression on both cold-activated and warm-suppressed sensory fibers. **B**. The ratio of time spent in nest by TRPM8 -/- virgins during cohousing with C57Bl/6N dams and pups. **Ci**. The ratio of time spent co-nesting. **Cii**. Time co-nesting by TRPM8 -/- vs C57Bl/6N virgins. **Di**. Ratio of shepherding. **Dii**. Shepherding ratio by TRPM8 -/- vs C57Bl/6N virgins. **Ei**. Nest area. **Eii**. Nest area built by TRPM8 -/- vs C57Bl/6N virgins. **Fi**. Pup retrieval success. **Fii**. Correlation between retrieval latency and ambient temperature. **Fiii**. Pup retrieval latency when relief nest is provided. **Fiv**. Latency of pup retrieval by TRPM8 -/- vs C57Bl/6N virgins. *p < 0.05, **p < 0.01, ****p < 0.001. Shaded area in Fii represents 95% confidence intervals.

161

162   Temperature does not affect pup retrieval success in TRPM8 -/- mice (**Figure 2Fi**, Fisher's

163   exact test, p = 0.315). Similarly, retrieval latency does not linearly correlate with ambient

164   temperature (**Figure 2Fii**, Spearman correlation, r = 0.23, p = 0.218, N = 30). However, we find

165   that the presence of a 'relief' nest in the CT but not WT condition speeds up retrieval in TRPM8 -

166   /- virgins similarly as in C57Bl/6 virgins (**Figure 2Fiii**, Wilcoxon test, CT, p = 0.007; WT, p =0.562).

167   Moreover, a genotype comparison shows no difference in retrieval latency between TRPM8 -/-

168   and C57Bl/6N virgins, as both show similar patterns of temperature modulation (**Figure 2Fiv**, two-

169   way ANOVA main effect of temperature F(3,55) = 4.87, p = 0.004, N = 63).

170   Thus, TRPM8 -/- mice have deficits in maternal behavior adaptation to both cold
171   temperatures (time in nest, shepherding), as well as thermoneutral and warm temperatures (nest
172   building). TRPM8 -/- mice have minimal disruptions of temperature-dependent pup retrieval,
173   indicating that this behavior could rely on additional mechanisms other than temperature
174   detection. Indeed, we confirmed prior findings showing that cold modulates pup vocalizations
175   (**Figure S2**), vocally communicated triggers of maternal pup retrieval behavior [20, 33, 34]. Our
176   findings validate a role for thermosensation in specific aspects of maternal thermoregulatory
177   behavior.

178

179   **Ambient temperature modulates activity of hypothalamic AVP+ neurons**
180   Temperature information travels from peripheral sensory fibers to spino-ponto-hypothalamic
181   circuits that mediate physiological and behavioral self-thermoregulatory responses [3]. PVN is
182   one of the hypothalamic nuclei important for thermoregulation [35-37]. PVN also hosts OT and
183   AVP expressing neurons, that have been linked to maternal behavior in both dams and virgin
184   female mice [20-22, 26, 31, 32]. To determine if ambient temperature modulates activity of OT+
185   and AVP+ neurons in PVN, we investigated the expression of c-fos immediate early gene. Virgin
186   females were exposed to one of the four temperature conditions for four hours, and then the
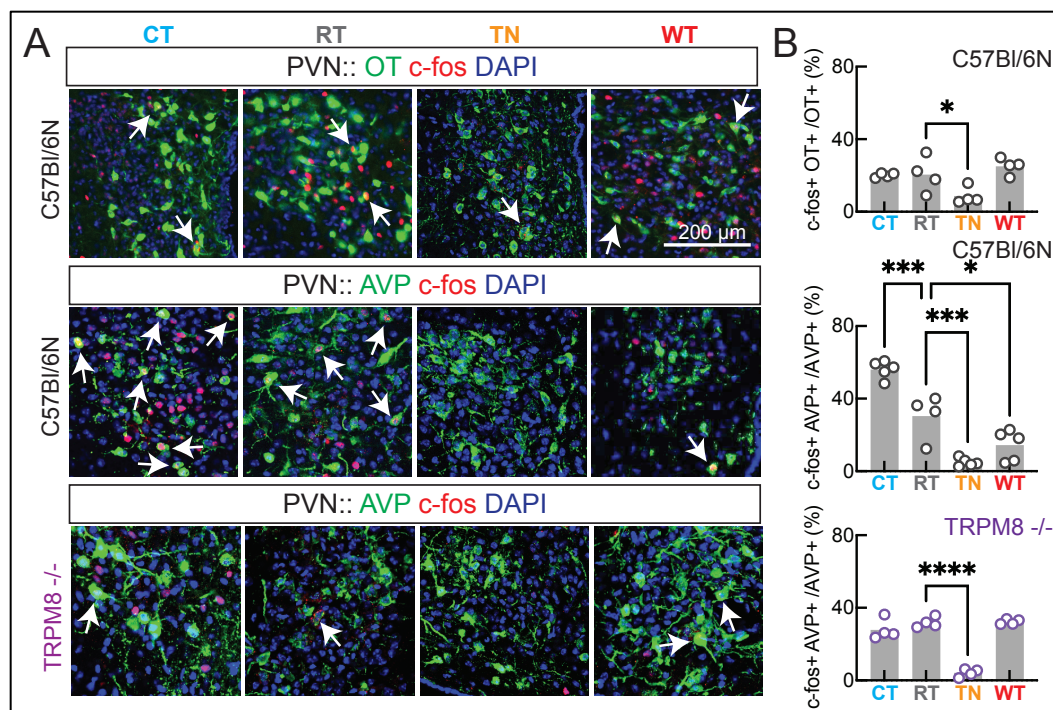
Figure 3: Temperature modulation of c-fos expression in PVN neuroendocrine cells in C57Bl/6N and TRPM8 -/- virgin female mice. **A.** Representative images showing c-fos, OT+ and AVP+ after incubation at different temperatures. **B.** Quantification of c-fos expression in neuroendocrine cells. *p < 0.05, ***p < 0.001, ****p < 0.0001.

187 brains were collected for c-fos and peptide immunostaining (**Figure 3A**). We find an effect of

188 temperature on c-fos expression in PVN-OT+ neurons that was driven by suppressed expression

189 at TN (**Figure 3B**, one-way ANOVA F(3,12) = 5.46, p = 0.013, N = 16; Dunnett's test for RT vs

190 CT, p = 0.999; RT vs TN, p = 0.041; RT vs WT, p = 0.582).

191 PVN-AVP+ neurons showed more profound responses to ambient temperature, as they

192 were activated at CT and suppressed at TN and WT (F(3,15) = 43.52, p < 0.0001, N = 19; RT vs

193 CT, p = 0.0004, RT vs TN, p = 0.0004, RT vs WT, p = 0.016). Importantly, both the activation of

194 PVN-AVP+ neurons at CT and their suppression at WT were absent in TRPM8 -/- female mice

195 (F(3,12) = 60.65, p < 0.0001, N = 16; RT vs CT, p = 0.291; RT vs TN, p < 0.0001, RT vs WT, p =

196 0.994). These findings indicate that PVN-AVP+ neurons could mediate thermosensation-driven

197 maternal thermoregulatory behaviors.

198

199 **Temperature responsive structures downstream of PVN-AVP+ cells**

200 To investigate if PVN-AVP+ neurons could play a role in maternal thermoregulatory behaviors,

201 we started by investigating whether they communicate synaptically with structures that play a role

202 in maternal care and respond to changes in ambient temperature. We studied this in two steps.

203 First, we mapped anterograde trans-synaptic connections from PVN-AVP+ neurons. For this, we

204 injected in the PVN of AVP-IRES-Cre mice a modified herpes virus that transduces TdTomato

205 fluorophore in Cre-expressing cells and then crosses the synapses to post-synaptic neurons. After

206 48 hrs of incubation we identified labeled cells in several structures that contribute to maternal

207 behavior: medial preoptic area (MPO, **Figure 4Ai**), ventromedial hypothalamus (VMH, **Figure**

208 **4ii**), ventral tegmental area, dorsal raphe, and periaqueductal gray (**Table 1**).

209 Second, we performed whole brain immediate early gene activity mapping following

210 exposure to different temperatures. Virgin females were incubated alone at one of the four

211 different temperatures for one hour, and after collection their brain was cleared, stained for Npas4

212 immediate early gene and imaged with light-sheet microscopy (**Figure 4B**). We calculated a

213 fluorescence index to identify brain regions that had changes in Npas4 expression at CT and WT

214 compared to RT condition ((F_{CT} − F_{RT})/(F_{CT} + F_{RT}) and (F_{WT} − F_{RT})/(F_{WT}+F_{RT})). Several brain

215 regions were activated or suppressed by exposure to CT or WT (**Figure 4Bi, Bii,** uncorrected one

216 sample t-test, H0 = 0). Few of these regions that receive PVN-AVP synaptic input (**Figure 4A**)

217 were activated by changes in temperature: MPO and VMH were activated at both CT and WT,

218 whereas CEA was activated only at CT. These findings indicate that PVN-AVP+ neurons project

219 to brain structures that respond to changes in ambient temperature, and indicate potential

220 mechanisms supporting maternal thermoregulatory behavior. Given our findings that only CT
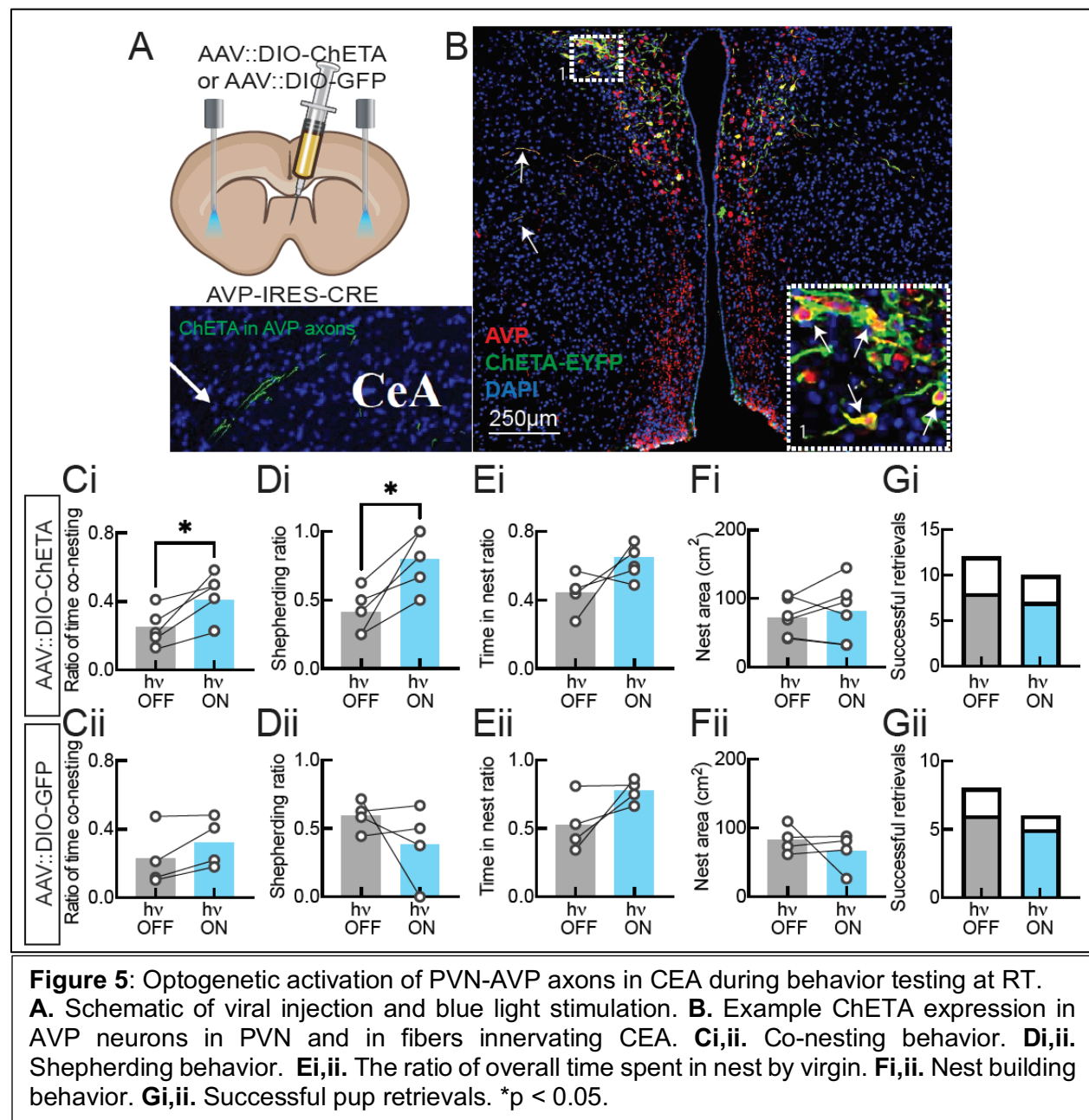
activates PVN-AVP+ cells, we next focused on PVN-AVP+ projections to CEA, which was specifically activated at CT and which previously received limited attention in the context of maternal behavior.
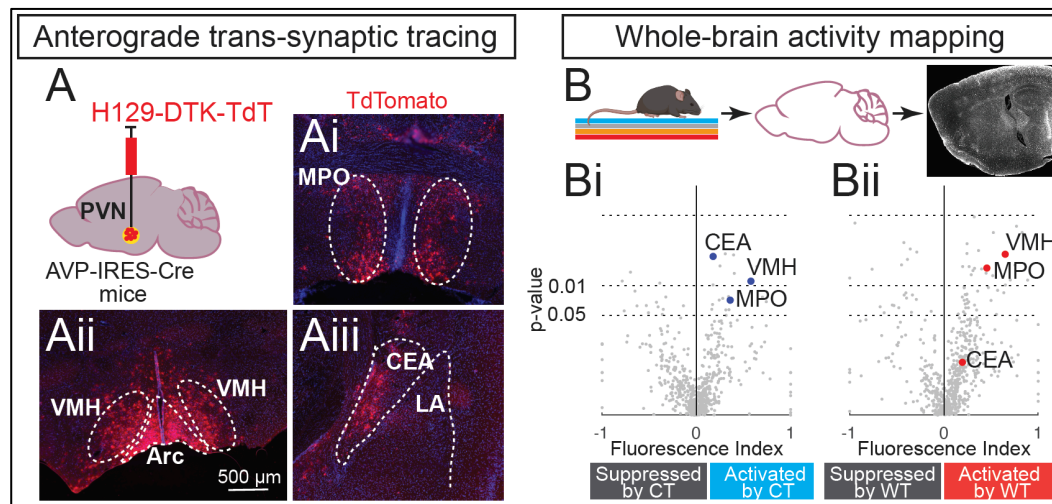
Figure 4: Temperature modulation of immediate early gene expression in brain regions receiving PVN-AVP synaptic input. **A-Aiii.** Experimental diagram and representative images showing anterograde trans-synaptic tracing from PVN-AVP+ neurons. **B-Bii.** Quantification of whole-brain activity mapping for Npas4 immediate early gene.

PVN-AVP+ fibers in central amygdala support maternal thermoregulatory care

To determine if vasopressin innervation of CEA could contribute to maternal thermoregulation, we optogenetically activated PVN-AVP+ fibers in the CEA and tested maternal behavior at RT (**Figure 5A,B**). In AVP-IRES-Cre virgin females transducing Cre-dependent channelrhodopsin-2 E123T (ChETA) in PVN, we found increased time co-nesting and enhanced shepherding behavior after receiving blue light stimulation in CEA (hv ON) compared to the control condition (hv OFF) (**Figure 5C**i: paired t-test t(df) = 3.86, 4), p = 0.018, N = 5; **Figure 5Di**: t(df) = 3.89 (4), p = 0.017, N = 5). These behaviors were not changed by blue light stimulation in animals transducing a control virus (**Figure 5Cii**: t(df) = 2.45 (3), p = 0.091, N =4; **Figure 5Dii**: t(df) = 1.14 (3), p = 0.334, N = 4). Activation of PVN-AVP+ fibers in CEA only modulated co-parenting behaviors (co-nesting and shepherding), whereas individual maternal behaviors (overall time in nest, pup retrieval, and nest building) were not changed by stimulation (**Figure Ei**: t(df) = 2.27 (4), p = 0.085, N = 5; **Figure 5Fi:** t(df) = 0.75 (5), p = 0.486, N = 6; **Figure 5Gi:** Fisher's exact test, p > 0.99), similar as in control animals (**Figure 5Eii:** t(df) = 2.18(3), p = 0.116, N = 4; **Figure 5Fii:** t(df) = 0.75(3), p = 0.507, N = 4; **Figure 5Gii:** p > 0.99).

Figure 5: Optogenetic activation of PVN-AVP axons in CEA during behavior testing at RT. **A.** Schematic of viral injection and blue light stimulation. **B.** Example ChETA expression in AVP neurons in PVN and in fibers innervating CEA. **Ci,ii.** Co-nesting behavior. **Di,ii.** Shepherding behavior. **Ei,ii.** The ratio of overall time spent in nest by virgin. **Fi,ii.** Nest building behavior. **Gi,ii.** Successful pup retrievals. *p < 0.05.

DISCUSSION

We found that maternal behavior adapts to changes in ambient temperature, with cold increasing and warm temperature decreasing several measures of ethological pup-directed care. Similarly, cold activates and warm suppresses hypothalamic PVN neurons producing AVP, a neurohormone implicated in maternal behavior. Both behavioral and hypothalamic AVP+ activity patterns were dependent on thermosensory mechanisms. We show that PVN-AVP+ neurons

249   connect synaptically with brain structures responsive to changes in ambient temperature. Among

250   these, CEA is specifically responsive to cold, and optogenetic stimulation of PVN-AVP+ fibers in

251   CEA recapitulates the effects of cold temperature on co-parenting but not individual maternal

252   thermoregulatory behaviors.

253

254   **Maternal thermoregulation**

255   Generally, cold ambient temperature promoted behaviors that keep pups warm (nest building,

256   huddling in the nest, pup retrieval) whereas warm temperature decreased these behaviors. We

257   did not observe aggression behavior directed towards pups. Our results enrich the understanding

258   of rodent maternal thermoregulatory behaviors [6, 9, 10], and mirror epidemiological findings in

259   humans, where cold days associate with low and warm days with high numbers of child neglect

260   cases [1]. Maternal thermoregulatory behaviors do not appear to be dependent on mating,

261   gestational and lactation status, as both primiparous dams and nulliparous virgin surrogates

262   display similar, albeit not identical, temperature-driven adaptations in pup care (**Figure 1**). Our

263   data (not included in this manuscript) indicate that the estrus cycle does not significantly affect

264   maternal behavior adaptations to ambient temperature. However this aspect needs to be studied

265   further in light of recent findings that the ratio of gonadal hormones play an important role in

266   shaping maternal behavior when self-directed needs are pressing [38]. An important question that

267   we began addressing in this manuscript and are actively investigating further, is the extent to

268   which the described changes in behavior are driven by pup needs versus self needs. In the pup

269   retrieval experiment, if female mice were primarily motivated by maintaining their own thermal

270   comfort, they would spend more time in the relief nest resulting in longer pup retrieval latencies.

271   However, we observe the opposite effect, where the presence of the relief nest accelerates pup

272   retrieval in cold (**Figure 1Civ**). Similarly, cold ambient temperature drove females to build larger

273   nests only in the presence of pups and not when housed without pups (**Figure 1Div**). Together,

274   these data indicate that cold temperature motivates maternal behavior in female mice.

275   The described temperature-dependent adaptations in maternal care could have profound

276   consequences on offspring development and well-being [39-41]. In this context, it is important to

277   emphasize that our manuscript investigates maternal behavioral adaptations in response to

278   relatively acute changes in ambient temperature. Recent work showed that mouse

279   thermoregulatory physiological processes adapt during long-term exposure (days, weeks) to

280   extreme ambient temperature [42]. It remains to be determined if maternal care could undergo

281   similar acclimations during prolonged exposure to cold or warm ambient temperature.

282

Thermosensory mechanisms

Mechanistically, we found that maternal thermoregulatory behaviors depend in part on thermosensation, as they were disrupted in mice lacking the TRPM8 temperature receptor (**Figure 2**). This was particularly evident for co-parenting behaviors in the co-housing assay and for the nest building assay. Intriguingly, TRPM8-mediated thermosensation appeared to mediate the effects of cold in co-parenting assay and the effects of warm temperature on nest building. Prior work has shown that TRPM8 is expressed on both cold-excited and warm-suppressed sensory fibers [14], and our findings indicate that these distinct pathways may differentially modulate activity in brain structures supporting specific maternal behaviors.

Alternative mechanisms could partially account for maternal thermoregulatory behaviors. One such alternative is that maternal behavior adapts to communication signals from pups [43]. Mouse pups emit ultrasonic isolation calls that drive pup retrieval behaviors in dams and naïve virgin [20, 23, 33, 34]. We find that cold changes the rate and frequency of pup calls (**Figure S2**), confirming prior work on the effects of temperature on pup vocalizations [44, 45]. It is possible that ambient temperature changes additional types of pup communication signals, for example body odor. Prior work has shown that olfactory cues make an essential contribution to pup retrieval behavior [46, 47]. We found mixed effects of TRPM8 deletion on pup retrieval at different temperatures (**Figure 2Fi-iv**): only a trend for females to retrieve more and faster in cold, but a significant acceleration of pup retrieval when a relief nest is provided. Together, these data indicate that thermosensation is not the only mechanism supporting the effects of ambient temperature on pup retrieval behavior. Although the effects of pup communication signals on nest building are less well understood, it is possible that they also contribute to cold-motivated nest building, as TRPM8 -/- virgins made similar size nests as C57Bl/6 virgins when exposed to cold (**Figure 2Eii**).

Changes in local brain tissue temperature could represent an additional mechanism by which changes in ambient temperature shape maternal behavior. Compared to humans, mice have less tight control of their core body temperature [48, 49]. Specifically, when ambient temperature rises above thermoneutrality, core body temperature slowly increases [50]. Many temperature receptors are also expressed in internal organs, including the brain, and could induce changes in neuronal excitability [51]. TRPM8, however, is primarily expressed in sensory fibers, and has only very sparse expression in the brain [11, 52]. This justifies the use of global TRPM8 -/- mice. Nevertheless, to exclude the possibility that even such sparse brain expression of TRPM8 could be responsible for maternal thermoregulatory behaviors, future work should test mice in which TRPM8 is deleted specifically from sensory fibers.

Vasopressin modulation of maternal behavior

In prior research on maternal care, the paraventricular nucleus of the hypothalamus (PVN) emerged as one of the brain structures mediating nest building, pup retrieval and time spent in nest [20-23]. Within PVN, several neuropeptides have been shown to play important roles in maternal behavior, primarily OT and AVP. Although both AVP and OT have been implicated in a variety of social behaviors, AVP has been generally considered to play more important roles in males than in females, while OT has been more often associated with maternal behavior in females [8, 53-58]. Intriguingly, we find that in females changes in ambient temperature modulate c-fos immediate early gene expression in AVP+ more than OT+ cells in the PVN (**Figure 3**). This corroborates prior work showing that cold increases AVP mRNA transcripts in rat PVN [59, 60]. OT- and AVP-producing neurons in other brain regions could also be engaged by changes in ambient temperature. In exploratory data not included in this manuscript, we do not find substantial temperature-induced changes in c-fos expression in OT+ neurons from supraoptic and suprachiasmatic nuclei, however such changes could be present in other OT-producing structures that we did not probe here: e.g. bed nucleus of stria terminalis, accessory and tuberal nucleus [61, 62].

While temperature-modulation of AVP+ cell activity could be linked to physiological functions of the AVP peptide (e.g.: control of water balance, vasoconstriction) [63], it also supports changes in maternal behavior. Increased brain AVP concentrations promote pup retrieval and increase the time spent caring for pups [26], potentially by synapsing on *Galanin* neurons in the medial preoptic area (MPO), another major regulator of maternal care [27, 28, 30]. AVP has also been linked to nest building behavior, as increased AVP expression was associated with decrease nest building [31]. Although this relationship is surprising given our finding that PVN-AVP+ activity increases in cold temperature, it could indicate that the effects of AVP on nest building depend on the source and target of AVP release. For example, mice selectively bred as good nest builders had fewer AVP+ cells in the suprachiasmatic nuclei but not PVN [64].

PVN-AVP+ neurons project to the posterior pituitary to release AVP in the blood stream for peripheral actions such as vasoconstriction, and project centrally to many brain areas implicated in parental behavior: MPO, VMH, bed nucleus of stria terminalis, periaqueductal gray, ventral tegmental area and others (**Figure 4**) [27, 28, 30, 65-70]. Some of these structures, such as MPO and VMH, respond to both increases and decreases in ambient temperature (**Figure 4)**, by receiving projections from temperature-responsive neurons within the ponto-hypothalamic pathway [5, 18, 71-74]. As such, these structures could contribute to maternal thermoregulatory behavior independent of AVP neuromodulation. Future work will directly test this possibility by

351   manipulating AVP terminals in these brain structures. In our current study, we elected to focus on

352   the CEA, a structure that receives PVN-AVP synaptic input and also responds to ambient cold

353   but not warm temperature (**Figure 4**). CEA receives direct projections from PVN-AVP+ neurons

354   (**Figure 5C**) [75] and also expresses vasopressin receptors 1a in both rodents and primates [76-

355   78]. We optogenetically activated bilateral PVN-AVP→CEA fibers in animals housed at room

356   temperature and tested maternal care. This manipulation mimicked the effects of cold on specific

357   maternal thermoregulatory behaviors, namely co-parenting behaviors, but not on pup retrieval

358   and nest building (**Figure 5**). Our findings indicate a role for CEA in maternal care, a role that has

359   been minimally investigated in mice. A previous study in lactating rats showed that AVP

360   concentration in CEA increases in response to interaction with an adult virgin female, but it led to

361   increased aggression toward the virgin [32]. This finding is in apparent contradiction with our

362   results, where PVN-AVP→CEA stimulation increases co-nesting and shepherding between virgin

363   and dam (**Figure 5Ci, Di**). However, species-specific differences in co-parenting strategies could

364   clarify the role of AVP in CEA. Whereas female mice typically nest communally, with dams and

365   other females rearing pups together, rat females rarely nest communally and preferentially with

366   co-reared sisters [79]. Thus, AVP in CEA could play a role in enhancing the species-specific co-

367   parenting strategy.

368   .

369   **MATERIALS AND METHODS**

370   **Animals**

371   Wildtype timed pregnant and virgin (nulliparous) adult female mice (C57Bl6N) were obtained from

372   Taconic Laboratories. TRPM8$^{-/-}$ homozygous mice were generated in the laboratory of Dr. David

373   Julius and obtained from The Jackson Laboratories (stock #008198). These mice have a mixed

374   C57Bl/6N and J background. AVP-IRES2-Cre heterozygous mice were generated in the Allen

375   Institute for Brain Science by Dr. Hongkui Zeng and obtained from The Jackson Laboratories

376   (stock # 023530). Experiments were performed with primiparous dams at postnatal day 1-14 and

377   virgin adult female mice of 8-12 weeks of age. Mice were housed at $22 \pm 2°C$, except when other

378   temperature was indicated in the text. Animals were housed under a 12-h light/dark cycle (lights

379   on at 8:00 a.m.), and had free access to food and water. Newborn mice were allowed to freely

380   breastfeed from their respective dams. Dams are single housed with their litter of pups until

381   weaning age of (21 days). Young virgin females are group-housed unless specifically stated in

382   the test for behavioral testing purpose. The animal care and experimental procedures were

383   carried out in accordance with the NIH guidelines and approved by the Rutgers State University

384   of New Jersey Institutional Animal Care and Use Committee (IACUC).

Statistical Analysis

The software used for data processing was Microsoft excel. The software used for all analysis was GraphPad Prism versions 9 and 10. For all datasets, we excluded outliers by using the ROUT method (with Q = 1%). That was followed by assessing the normal distribution of the sample Shapiro-Wilk test. Samples that had normal distribution were analyzed with parametric tests, and those that didn't have normal distribution were analyzed with non-parametric tests.

Temperature treatments

All the temperature conditions in this work were within the non-noxious temperature ranges. The four temperature conditions were achieved with artificial cooling or heating of the testing apparatus. Warm temperature (WT, 35-38°C) and thermoneutral (TN, 29-32°C) conditions were created by placing a heating pad (RIOGOO Pet Heating Pad with Auto Power, Amazon.com) under covered cages (NOLDUS phenotyper® 4500 cage with floor area of 45cm x 45cm (18" x 18"), or the innocage®, (INNO VIVE) 14.7"L x 9.2"W x 5.5"H (maximum), (37.3 x 23.4 x 14.0 cm (maximum). The cold temperature (CT) condition (15-16°C) was created by setting a refrigerated chamber or insulated space to 15°C. The room temperature condition (22-25°C) was the ambient room temperature during the experiment. When the ambient room temperature (RT) in the observation room was below 21°C the heating pad was place under covered cages to achieve temperatures between 22-25°C. The temperature gradient conditions used in the pup retrieval assay were achieved by placing a cooling pack in WT condition and placing an activated hand warmer (HotHands) in the CT condition under the nest area to reach TN temperature (29-32°C).

Behavior assays
Maternal behaviors

The sequence of behavior assays performed were co-housing assay, followed by pup retrieval and lastly nest building. The co-housing assay was performed first to prime the virgin females to acquire maternal behavior from interactions with dams and pups. Although all animals underwent co-housing for priming, only in a subset of animals we videotaped this procedure for behavioral analysis.

Co-housing assay: The pregnant females and naive virgins were kept separate cages until used for this behavior assay. After the pregnant female underwent parturition becoming dams, the assay was conduction between postnatal day one through seven (P1-P7). The naïve virgins were approximately eight to twelve (8-12) weeks old during the assay. Co-housing of a naive virgin (young female) with a dam and her litter was conducted for 4-6 hours per day for 3-4 consecutive

419   days. Each pair of naive virgin and dam with her litter of 3 pups were exposed to a temperature

420   conditions randomly assigned. The naive virgins' fur was bleached and dye pink with semi-

421   permanent dye and marked on their tails for identification. Animals were transferred from their

422   home cage to the phenotyper cage (NOLDUS) for the assay and were returned to their home

423   cage after completing the assay. The nesting materials from the mother's home cages was also

424   placed in the phenotyper cage. The floor of the phenotyper cage was covered with abundant

425   bedding material. We added food pellets in the feeder and a water bottle. We first placed the dam

426   and her postnatal day 1 (P1) litter in the cage. The assay was recorded using Color GigE cameras

427   on the Ethovison software (NOLDUS). The behaviors were manually scored using BORIS version

428   7.7 through 8.4 [80]. Shepherding behavior (pursuing the other adult animal toward the nest) was

429   identified as previously described [23], if the animal being chased entered the nest. Pursuits away

430   from nest were all pursuits that did not end up with the chased animal in the nest. To be included

431   in the calculation of the 'Shepherding ratio', the animals had to have a minimum 2 pursuit.

432   **Pup retrieval assay:** This behavior assay was performed in dams and young virgin female

433   subjects adapted from Dr. Catherine Dulac's work [81]. The young virgin females were housed

434   individually for 18-24 hours prior to pup retrieval assay. Testing was performed with two (2) pups

435   aged p3-7 in the home cage with the home nest material. A 30 min habituation period to the

436   ambient temperature condition was performed before the testing commenced. The two C57BL/6N

437   pups were removed from the nest and placed in different corners opposite the nest. Each trial had

438   a ten (10) minutes limit, five minutes to retrieve each pup. For the 'relief nest experiment, the

439   conditions were as follow. At CT, the nest area was warmed to thermoneutrality (T°nest = 28.12

440   ± 2.31) by placing a handwarmer under the nest., and at WT the nest area was cooled (T°nest =

441   16.41 ± 1.38) using a cool pack under the nest. The test was recorded using logitech c-290

442   webcam. The behaviors were scored manually using BORIS version 7.7 through 8.4.

443   **Maternal nest building assay:** Maternal nest building was performed using dams and naïve

444   virgins. Dams with their litter were exposed to all the temperature conditions randomly assigned

445   for 4 hours per day for 3-4 consecutive days within the second week postpartum (P8-P14). Each

446   dam with culled litter to 5 pups was placed in an innocage® (INNO VIVE) which was covered

447   during the recording. In the cage adequate bedding, food, and hydrogel (a substitute for water)

448   was provided. However, nest material was not provided. The recording began with the last minute

449   of the acclimation stage. After ten (10) minutes of acclimation, a square cotton nestlet (Ancare®)

450   was placed in a cage. The recording was ended after four hours. When maternal nest building

451   was performed with naïve virgins, they are grouped housed with other virgins and each tested for

452   maternal nest building with 5 pups. The nestlet was removed and flattened on a black surface

453 and imaged along with a ruler to calibrate the pixels for calculating the area and perimeter. The

454 nest structure was scored based on how broken the material was. The nest score of 0 for 'Barely

455 broken up', 1 for '10-20% slightly broken up', 2 for '20-50% partly broken up', 3 for '50-80% partly

456 broken up' and 4 for '80-100% completely broken up'.

457

458 **Pup behaviors**

459 **Pup isolation ultrasonic vocalization recording:** Pups aged P2-7 were each isolated from

460 home cage with dam and rest of the litter to a beaker lined with bedding set at the four temperature

461 conditions. The AVISOFT Avisoft-Bioacoustics CM16/CMPA microphone was placed around 3-4

462 inches above the base. The pup's ultrasonic vocalizations (USVs) were recorded for 120 seconds.

463 The pups were marked and returned to the home cage. This was repeated until all the pups were

464 recorded for multiple litters of pups. The audio files were analyzed using the MUPET algorithm

465 [82].

466
467 **Adult-only behavior**

468 **Virgin nest building assay in the absence of pups:** Grouped housed naive C57Bl/6N virgins

469 were separated and randomly selected to a nest building assay. They were each placed in a

470 single housed testing apparatus (innocage® (INNO VIVE)) which was covered during the

471 recording. In the cage adequate bedding, food, and hydrogel (a substitute for water) was provided.

472 However, nest material was not provided. After ten minutes of acclimation the recording began

473 with a square cotton nestlet (Ancare®) was placed in a cage. No pups were present. The

474 recording ended after four (4) hours. The nestlet was removed and flattened on a black surface

475 and imaged along with a ruler to calibrate the pixels for calculating the area and perimeter. The

476 nest structure was scored and analyzed same as in the maternal nest building assay.

477

478 **Immediate early gene expression assessment**

479 **Temperature exposure assay**: Grouped housed naive C57Bl/6N virgins were habituated to

480 handling and single housing in a testing apparatus (innocage® (INNO VIVE)) for four (4) hours

481 over four days. On the fifth day (test day), they were each randomly selected to the same test

482 apparatus set at one of the four temperature conditions. The temperature exposure lasted for four

483 hours. The mice were perfused, and brain tissue was collected within five-fifteen minutes from

484 the end of the temperature exposure.

485 **Immunohistology**: Brain tissues were collected after transcardial perfusion with 1 X PBS,

486 followed with 4% paraformaldehyde (PFA). Tissues were further preserved in a paraformaldehyde

487   solution for 1–2 h at 4 °C, followed by cryopreservation of tissue in 15% and then 30% sucrose

488   solution at 4 °C. The tissues were embedded in OCT solution and sectioned at 20 µm thickness

489   with a cryostat then mounted on positively charged slides. Sections were blocked and

490   permeabilized with 5% goat serum solution with 0.1% triton-X in 1x PBS for 1 h at room

491   temperature. Primary antibodies were diluted in 1% goat serum and 0.01% triton-X in 1x PBS

492   solution and then applied for 24hr - 48hr at 4 °C. We used a mouse anti-oxytocin antibody (1:100,

493   a gift from H. Gainer at national Institutes for Health) [83], mouse anti-vasopressin antibody (a gift

494   from H. Gainer at national Institutes for Health, 1:100) [83], rabbit anti-c-fos (Cell Signaling

495   Technology, 1:500), a chicken anti-GFP (Aves, 1:500), and a rabbit anti-tDT (Takara, 1:500)

496   antibody. The sections were washed in 2 to 3X in PBS solution. Then, the appropriate solutions

497   of fluorophore-conjugated secondary antibodies were applied for 1.5 h at room temperature. All

498   secondary antibodies were from Jackson ImmunoResearch and used at 1:1000 (Anti-Rabbit Goat

499   Cy3, Anti-Mouse Goat Cy2, and Anti-Chicken Goat Alexa Fluor 488). The sections were washed

500   in 2 to 3X in PBS solution again. After which, all sections were counterstained with DAPI (4',6-

501   diamidino-2-phenylindole). Slides sealed with Fluoromount-G™ Mounting Medium (Invitrogen)

502   and a glass cover coverslip. Slides were imaged using a Nikon A1R+ HD Confocal Microscope

503   and a Nikon Ti2 HCA Inverted Fluorescence Microscope at Rutgers New Jersey Medial School,

504   Cellular Imaging & Histology Core. The confocal microscope is equipped with four solid-state

505   lasers line (405, 488, 567, and 637nm) and the appropriate filter cubes. The acquired images

506   were analyzed using ImageJ and QuPath, version: 0.4.3 (University of Edinburg) for colocalization

507   of OT+ and AVP+ cells with c-fos expression. Immunohistochemistry was also performed for the

508   examination of anterograde tracing, and confirmation of viral expression in the optogenetic

509   stimulation studies.

510   **Whole brain activity mapping:** C57Bl6 virgin female subjects were each exposed to one of the

511   four temperature conditions for one hour. Within 5-15 minutes after the brain tissue were collected

512   after standard 4% PFA transcardial perfusion followed by an overnight incubation in 4% PFA at

513   4°C. Samples were washed three times for 1 hour each with PBS and stored in PBS + .02%

514   Sodium Azide at 4°C for storage and shipment. The whole brain tissue samples were sent to

515   Transluscence Biosystems for whole brain activity analysis. The sample were cleared using

516   iDISCO method. Then, probed using immunoglobin against markers for neural activity (immediate

517   early genes, c-fos and Npas4). Images were acquired using light sheet imaging on ZEISS

518   Lightsheet Z.1 and Lightsheet 7 microscopes using our Mesoscale imaging System™. This was

519   followed by machine learning-based image analysis with data registration using the Allen Brain

520 Atlas as reference. The data was analyzed based on fold change in cold and warm temperature
521 conditions relative to the control temperature conditions (RT).

522 **Anterograde tracing:** To examine the brain regions that receive inputs from PVN-AVP cells,
523 stereotaxic viral injections were performed in virgin female AVP-IRES-Cre mice. Mice were
524 weighed, then anaesthetized with 1.5–2.5% isoflurane (adjusted based on reflexes and breathing
525 rate during surgery), placed into a stereotaxic apparatus (NeuroStar, Germany). Local anesthetic,
526 Bupivacaine, was injected subcutaneously prior to incision to access the skull. Unilateral
527 craniotomies performed over left PVN (coordinates: anteroposterior (AP): -0.70 mm from Bregma;
528 mediolateral (ML): +0.18 mm from the midline, dorsoventral (DV): −4.7 mm). Cre-inducible,
529 transsynaptic herpes simplex viruses 1 strain, H129ΔTK-TT (National Institute of Health (NIH)-
530 The Center for Neuroanatomy with Neurotropic Viruses (CNNV)) was injected at a depth of 4.7
531 mm with a 5 µl Hamilton syringe and a 33 gauge needle [84]. The virus was injected into PVN at
532 0.1 µl min^{-1} for a final injection volume of 0.5µl. The craniotomy was sealed with a silicone
533 elastomer (World Precision Instruments), the skin sutured and antibiotic ointment was applied.
534 Post surgery, analgesia, buprenorphine extended-release injectable suspension was given to
535 subjects at the appropriate dose. Brain tissue samples were collected from subjects after
536 transcardial perfusion 48 hours after surgery to examine anterograde tracing viral expression.
537 Tissue was processed for immunohistochemistry and images acquired with fluorescence
538 microscopy.

539

540 **Optogenetic stimulations: surgery (injections and implantation) and behavior protocol.**
541 For optogenetically manipulating PVN-AVP→CEA projections, stereotaxic viral infections and
542 fiber implantations were performed in virgin female AVP-IRES-Cre mice. Subjects were weighed,
543 then anaesthetized with 1.5–2.5% isoflurane placed into a stereotaxic apparatus (NeuroStar,
544 Germany). Local anesthetic, Bupivacaine, was injected subcutaneously prior to incision to access
545 the skull. Bilateral craniotomies performed over left PVN (coordinates: AP: -0.70 mm, ML:
546 +0.18 mm, DV: -4.7mm) and right PVN (coordinates: AP: -0.70 mm, ML: -0.4 mm, DV: −4.7 mm).
547 Cre-inducible engineered opsin expressing AAV, AAV9-Ef1a-DIO ChETA-EYFP, (addgene) or
548 AAV pCAG-FLEX-EGFP-WPRE-AAV9 (addgene) as control was injected with a 5 µl Hamilton
549 syringe and a 33 gauge needle [85]. The virus was injected into PVN at 0.1 µl min^{-1} for a final
550 injection volume of 0.5µl. Craniotomy for optic fibers and supporting bone screw were drilled.
551 Bilateral implantation of 5mm optic fibers (Doric Lenses) were inserted above the left CeA
552 (coordinates: AP: -1.70 mm, ML: +2.6 mm, DV:-4.1mm) and above right CEA, (coordinates: AP:
553 -1.70 mm, ML: -2.8 mm, DV:-4.05mm) at a speed of 10µm sec^{-1}. The craniotomy was gently

554 sealed with a silicone elastomer (World Precision Instruments). The implanted optic fibers were
555 secured to the skull using dental cement (C&B Metabond). We applied antibiotic ointment and
556 sutured the scalp. Immediately after surgery we administered analgesia, buprenorphine
557 extended-release injectable suspension. Animals were used for experiments after three weeks to
558 allow for viral opsin (ChETA) expression in PVN-AVP axonal terminals.

559 To perform optogenetic stimulations, a blue light laser (OptoEngine LLC.) was connected
560 with patch cord and sleeves (Neurophotometrics LLC.) to the optical fibers implanted above CEA
561 of the implanted AVP-ires-Cre virgin mice. At the beginning of each behavior assay, we stimulated
562 with 5ms light pulses, delivered at 20 Hz with laser power between 7 to 15 mW lasting for 30
563 minutes. Then, the virgin was disconnected to continue the behavior assay. The 'light off'
564 experiments were conducted with the laser turned off. Each animal was tested under 'light on'
565 and 'light off' conditions on separate days, and across mice we alternated the order of on and off
566 days. After behavior studies were concluded brain tissue samples were collected from subjects
567 after transcardial perfusion 48 hours after surgery to examine viral expression and implantation
568 accuracy. The brain tissues were processed for immunohistochemistry as described above, and
569 images acquired via fluorescence microscopy.

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579

580 **AUTHOR CONTRIBUTIONS**
581 ZA and IC designed the experiments, analyzed data and wrote the manuscript. ZA conducted the
582 experiments. RO had a major contribution to histology data collection. DBG, ZZM, HKH, BS and
583 NDA contributed to behavioral data analysis. JSR analyzed the whole-brain activity data,
584 contributed to the general design of the study, and edited the manuscript.

585

586 **DECLARATION OF INTEREST**
587 The authors do not have a conflict of interest.

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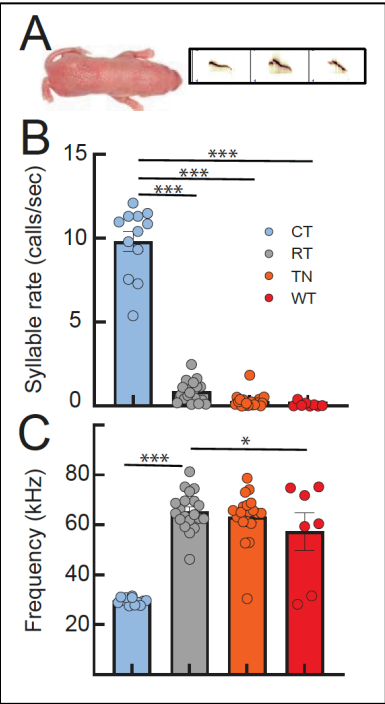779 938-950.

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782 **SUPPLEMENTARY FIGURES AND TABLES**

783
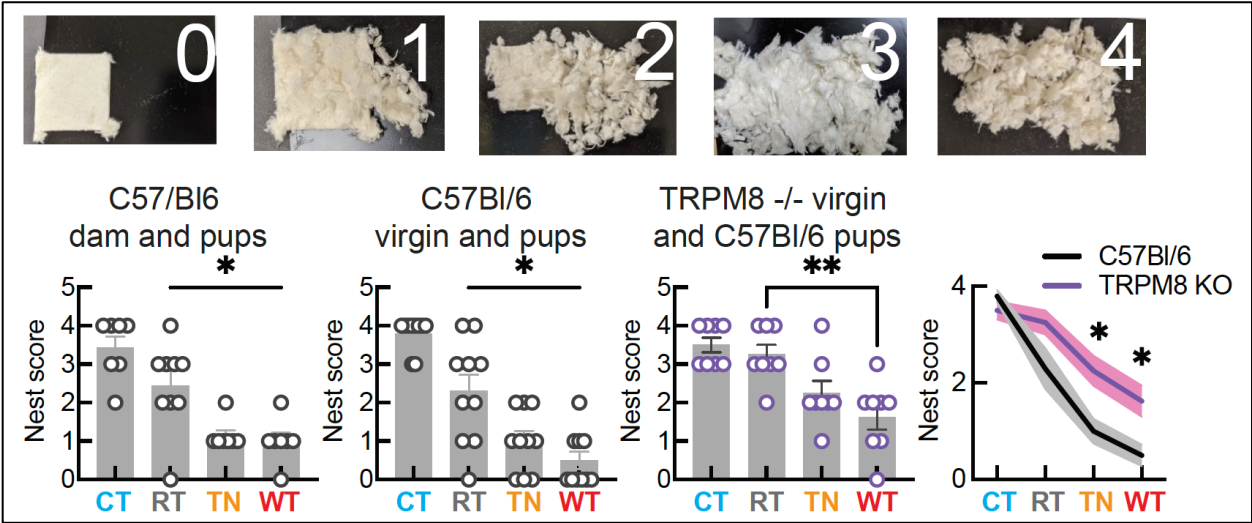
Figure S1: Nest score. Top, example nestles scored with different scores. Bottom, quantification of nest scores. *, p<0.05; **, p<0.01.

Figure S2: Pup isolation calls in response to changes in ambient temperature. A. Example spectrogram of isolation calls. B. Quantification of syllable rate. C. Frequency of isolation calls. *, p<0.05; ***, p<0.005.

784

No.	Regions with TdTomato+ cells	Abbreviation	Laterality	Intensity
1	dorsal transition zone	DTr	Bilateral	+
2	ventral orbital cortex	VO	Bilateral	+
3	medial orbital cortex	MO	Bilateral	+
4	cingulate cortex, area 2	Cg2	Bilateral	+++
5	nucleus accumbens core and shell	Nac	Bilateral	+

6	anteroventral periventricular nucleus	AVPe	Bilateral	++	
7	lateral septal nucleus, ventral part	LSV	Bilateral	++	
8	mediodorsal thalamus	MD	Unilateral	++++	
9	paraventricular thalamic nucleus	PVA	Bilateral	+++	
10	suprachiasmatic nucleus	SCh	Bilateral	++	
11	paraventricular nucleus of the hypothalamus	PVN	Bilateral	+++	injection site
12	nucleus of the stria medularis	sm	Unilateral	++++	
13	anterior hypothalamic area	AH	Bilateral	+	
14	supraoptic nucleus	SO	Bilateral	++	
15	median preoptic area	MnPO	Bilateral	++	
16	bed nucleus of stria terminalis	STS	Bilateral	++++	
17	lateral hypothalamic nucleus	LH	Bilateral	++	
18	anterodorsal thalamic nucleus	AD	Unilateral	++++	
19	medial preoptic area	MPA	Bilateral	+	
20	CA2 of hippocampus	CA2	Bilateral	+	
21	central amygdala	CeA	Bilateral	++	
22	arcuate nucleus	Arc	Bilateral	+++	
23	retrochiasmatic area	RCh	Bilateral	++	
24	median eminence	ME	Bilateral	+	
25	lateral amygdala	La	Bilateral	+	
26	enthorinal cortex	Ent	Bilateral	+	
27	ectorhinal cortex	Ect	Bilateral	+	
28	primary auditory cortex	Au1	Bilateral	+	
29	secondary visual cortex	V2	Bilateral	++	
30	lateral mammilary nucleus	LM	Bilateral	++	
31	CA3 of posterior hippocampus	CA3	Bilateral	++	
32	peri-aqueductal gray	PAG	Bilateral	++	
33	dorsomedial hypothalamic nucleus	DMH	Bilateral	+	
34	posterior hypothalamus	PH	Bilateral	+	
35	ventral tegmental area	VTA	Bilateral	++	
36	substantia nigra pars compacta	SNC	Bilateral	+	
37	dorsal raphe nucleus	DR	Bilateral	++++	
38	A5 noradrenaline cells	5ADr	Unilateral	+	
39	interfascicular nucleus	IF	Bilateral	+	
40	retromammilary nucleus	RM	Bilateral	+	
41	subgeniculate nucleus of prethalamus	SubG	Bilateral	+	
42	lateral parabrachial nucleus	LPB	Bilateral	++++	

Table 1: Quantification of anterograde trans-synaptic tracing from PVN-AVP+ neurons (unilateral injections). + = 0.1-100 cells in 1×10^6 px^2 ; ++ = 100.1-200 cells in 1×10^6 px^2; +++ = 200.1-400 cells in 1×10^6 px^2; ++++ = 400.1-above cells in 1×10^6 px^2.