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In humans, anticipatory stress involves activation of the limbic–hypothalamic–pituitary–adrenal axis, which releases stress hormones such as cortisol in response to an impending stressor. Conditioning of the stress response to anticipate and prepare for future challenges is a hallmark of adaptation. It is unknown whether human infants in the first year of life have developed the neural circuitry to support the anticipation of stressful events in an attachment context. Here, we show that human infants at six months of age produce an anticipatory stress response, as indicated by the release of stress hormones, when re-exposed after 24 h to a context in which they demonstrated a stress response to a disruption in the parent–infant relationship. Although infant stress response (cortisol elevation) was greater to the stressful event (parent unresponsiveness) than to the second exposure to the stress context (room, chair, presence of parent and experimenter, etc.), it was greater in the stress group than in the control group on both days. Results suggest that human infants have the capacity to produce an anticipatory stress response that is based on expectations about how their parents will treat them in a specific context.

Keywords: anticipation; stress; cortisol; attachment; memory; parenting; infancy

1. INTRODUCTION

Anticipating future events is adaptive for young human infants because it allows them to protect themselves from physical harm by avoiding threats such as rapidly approaching objects [1] and visual cliffs [2]. In addition, it supports their developing capacity to attend to the intentions of others [3] and form expectations about how others will treat them [4]. Although many cognitive capacities permit the infant to anticipate events based on memory or logical reasoning, it remains unknown whether the infant limbic–hypothalamic–pituitary–adrenal (LHPA) axis can be conditioned to anticipate emotional disruptions in infants' relationships.

In the first six months of life, human infants show stress responses to relatively mild everyday perturbations such as taking a bath [5] as well as more novel events like undergoing a medical exam [6]. Infants also show stress responses to psychological stressors such as disruptions in their relationships [4]. For instance, when parents fail to respond quickly

and appropriately to the infant's emotional signals and instead show a neutral facial expression, infants become visibly upset and demonstrate a physiological stress response [7]. Infant stress responses rapidly diminish in response to novel events such as medical exams and learning tasks when repeated after a 24 h delay [6,8], suggesting that the infant learns that the experiences are not harmful to them and indicating that the stress response can be habituated in young human infants. However, it remains unknown whether infants experience stress conditioning that would allow them to mount a cortisol response in anticipation of a potentially stressful event.

We chose to introduce a psychological stressor—a relationship disruption—to determine whether infants would show anticipatory stress responses and whether this response would remain stable or undergo a decrease or an increase in stress reactivity. In other words, would infant stress response to this brief relational disruption (the still face) cause anticipatory anxiety, or a stress response, the next day?

2. MATERIAL AND METHODS

Participants consisted of 30 mother–infant dyads with infants six months of age (mean age = 24.2 weeks, s.d. = 1.1). The dyads were randomly assigned to either a stress group (the still-face task; $n = 15$) or to a control group (the responsive face-to-face task; $n = 15$).

On day 1, mothers were given an overview of the procedures, which described what they would be doing on both days. Following this, both infant groups interacted with their parents for 10 min. In the control group, parents and infants interacted as they normally would (in general, exchanging vocalizations, smiles, expression imitations, etc.). The inclusion of a control group insured that any changes in infant stress hormones would be a function of the emotional stress of the parent's unresponsiveness rather than the novelty of the laboratory or other confounds on days 1 and 2. In the unresponsive, still-face condition, the mother faced the infant, who was seated in a car seat placed on a table. The mother was instructed to look slightly above the infant's head and to maintain a neutral facial expression for 2 min. The still-face session consisted of the following interactions: a free play (2 min), still-face (2 min), second free play (2 min), second still-face (2 min) and a final free play (2 min). Three mothers momentarily broke the still-face to either adjust the posture of an infant who was slumping in the chair or to prevent an infant from chewing on a seatbelt.

After 24 h, the stress and control groups returned to the same room in the laboratory; however, neither the stress nor the control conditions were repeated on day 2. Instead, infants were seated on their parents' laps or by their sides for a duration equivalent to that of their task on day 1. Salivary samples were collected on both days from infants in order to analyse cortisol levels, which are a common measure of stress. The same experimenter collected the saliva samples on both days. Baseline was collected in the testing room; post-stress saliva samples were collected in the waiting room on both days.

To create the impression that infants would undergo another stress or control task on day 2, infants were brought to the same testing room, underwent the same initial procedures that led up to the start of the task on day 1, and were tested by the same experimenter on both days. To control for the effects of circadian rhythms on LHPA axis activity, time of testing was the same on both days (± 15 min). To evaluate whether infant affect levels changed during the study on days 1 and 2, the parents and infants were videotaped during testing.

(a) *LHPA activity*

To measure infant stress hormone responses, salivary samples were collected pre-task and at 20 min and 30 min post-task on days 1 and 2. Salivary samples were collected using cotton sorbettes. In order to ensure adequate volume, two sorbettes were used for each saliva sample. Experimenters placed the sorbettes under the infant's tongue for 45–60 s or until the sorbettes had collected sufficient volume. All saliva samples were stored in the investigating laboratory at $-40°C$ until assayed. On the day of the assay, salivettes were centrifuged for 10 min at 3000g at 4°C. All samples were assayed in
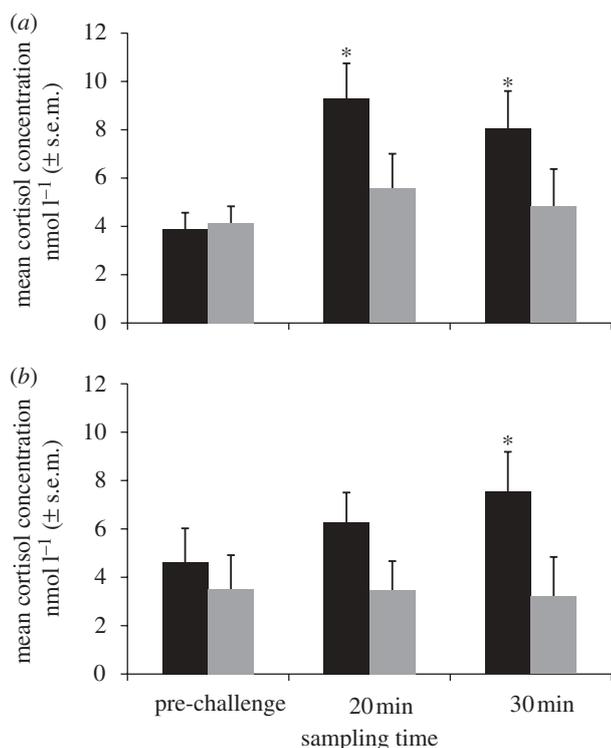
Figure 1. Cortisol responses on (*a*) day 1 and (*b*) day 2 in the stress and control groups, and error bars represent standard errors. Mean cortisol concentrations during pre-challenge, 20 min post-challenge, and 30 min post-challenge in the stress and control groups. The infants in the stress group showed significant elevations in cortisol concentrations in response to the still-face challenge on day 1 and in anticipation of a challenge on day 2. By contrast, the infants in the control group did not show an elevated response to the face-to-face challenge on day 1 and showed no cortisol response on day 2. Asterisk denotes $p < 0.05$ (between pre-challenge and 20 min or between pre-challenge and 30 min within group). Black bars, stress (still-face); grey bars, control (free-play).

duplicate using a salivary cortisol enzyme immunoassay kit (Salimetrics, State College, PA, USA). (Information about cortisol analysis, see electronic supplementary material.)

(b) *Affect*
Raters coded each 10 s interval on a scale from -3 to 3, with a rating of -3 indicating rhythmic crying for greater than 3 s and a rating of three indicating laughing greater than 2 s. The scores for 10 s intervals were averaged for each episode.

(c) *Time of day*
Each appointment on day 1 was scheduled at a time (mean = 11.55; s.d. = 1 h 38 min) that was convenient for the parent and did not interfere with scheduled feedings or nap times. These times were not significantly different between the stress (mean = 0.02) and control (mean = 11.48) groups ($F_{1,30} = 0.15$, $p = 0.701$). Time of day was entered as a covariate in the cortisol data analysis.

3. RESULTS
The cortisol results of the infant anticipatory stress experiment are presented in figure 1. Infants in the stress and control groups were included in the analysis. We evaluated whether group cortisol levels were equivalent using a repeated measures analysis of variance (ANOVA) with main effects of group (stress versus control), time (pre-challenge, 20 min, 30 min) on days 1 and 2. On day 1, there was a significant group × time interaction ($F_{2,56} = 5.34$, $p = 0.008$).

On day 2, there was group × time interaction ($F_{2,56} = 5.24$, $p = 0.009$) as well. Follow-up *t*-tests indicated an increase in cortisol concentrations from pre-challenge to 20 min post-challenge on both days (day 1: $t = 3.84$, $p = 0.002$; day 2: $t = 2.78$, $p = 0.015$) as well as from pre-challenge to 30 min post-challenge on both days (day 1: $t = 2.20$, $p = 0.045$; day 2: $t = 2.16$, $p = 0.048$). By contrast, the control group did not show elevations in cortisol concentrations during testing on days 1 and 2.

For the affect data, a one-way ANOVA indicated that there was a significant group × day interaction ($F_{1,27} = 5.30$, $p = 0.02$). Infants in the stress group showed a greater reduction in positive affect in response to the still-face condition compared with infants in the control group on day 1. On day 2, there was little change in affect in either the stress or control groups.

4. DISCUSSION
The findings show that infants as young as six months of age have memories of stressful events associated with a brief disruption in the parent–infant relationship, and that such memories persist for at least 24 h and manifest as anticipatory stress. Infants in the stress group showed a cortisol response not only to the challenge of parent unresponsiveness on day 1 but also to anticipation of this event on day 2. By contrast, infants in the control group did not show changes in cortisol concentrations either on day 1, during free play with their parents, or 24 h later, on day 2, during their second visit to the laboratory. Given that there were no differences in pre-task cortisol concentrations between the stress and the control groups on day 2, and given that cortisol is known to enter the saliva approximately 20 min after a potential stressor is perceived, the infants' anticipatory stress response appears to have been triggered after arrival in the laboratory where the testing took place.

Although infants in the stress group showed greater negative affect on day 1 than controls, we did not find any differences in affect on day 2. It should be noted that the infant cortisol response diminished from day 1 to day 2 in both groups of infants, which is consistent with previous research showing that the LHPA axis rapidly decreases to repeated challenges. A limitation of the study is that neither the parents nor the experimenters were blind to the experimental manipulation. Although it would be challenging, it is possible that this limitation could be addressed in a future study using live and recorded video.

The current study demonstrates that human infants have the capacity to produce an anticipatory stress response that is based on expectations about how their parents will treat them. This capacity to anticipate specific types of parent–infant interactions is remarkable for several reasons. First, it provides a unique window into the infant's emotional attachment to the caregiver and underscores the impact of the parent–infant relationship on the development of the stress system. Second, it suggests that the infant is neurobiologically equipped with a rather precocious set of social cognitive skills. A recent brain-imaging study, for

example, shows that the neural machinery which adults are known to use to share attention with others (the left dorsal prefrontal cortex) operates in infants as young as five months of age [9]. Whether the infant's sensitivity to disruptions in joint attention would similarly activate or differentially inhibit the same brain region would be an interesting question to pursue. Third, the current study suggests that infants are not only sensitive to relationship disruptions but can remember them, which is consistent with electro encephalogram studies of infant event memory in which greater electrical activity in frontal sites of the brain during encoding of novelty predicted better memory recall in nine month-old infants [10]. Finally, we can speculate that the infant's anticipatory stress response may enable the human infants to both anticipate and potentially adapt to changes in parenting.

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