Received: 2 February 2016 | Revised: 15 January 2017 | Accepted: 25 January 2017

DOI: 10.1002/dev.21506

WILEY Developmental Psychobiology

RESEARCH ARTICLE

Pubertal development and anxiety risk independently relate to startle habituation during fear conditioning in 8–14 year-old females

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Funding information
National Institute of Mental Health, Grant number: R01 MH097767

Abstract

Reduced habituation to aversive stimuli has been observed during adolescence and may reflect an underlying mechanism of vulnerability for anxiety disorders. This study examined the startle reflex during a fear-learning task in 54 8–14-year-old girls. We examined the relationship between mean startle, startle habituation, pubertal development, and two measures linked to risk for anxiety: behavioral inhibition system (BIS) and the error-related negativity (ERN). Puberty, BIS, and the ERN were unrelated to mean startle; however, each measure modulated startle habituation. Greater pubertal development was associated with reduced startle habituation across the CS+ and CS−. Higher BIS related to a larger ERN, and both were associated with reduced startle habituation specifically to the CS+. All effects were independent of each other. Findings suggest that puberty alters habituation of defense system activation to both threat and safety cues, and this is independent of risk for anxiety, which uniquely impacts habituation to threat cues.

KEYWORDS

behavioral inhibition system, ERP, error-related negativity, fear learning, puberty, startle habituation

1 | INTRODUCTION

Fear learning and extinction are key processes related to the development of anxiety disorders (Casey, Jones, & Hare, 2008; Liberman, Lipp, Spence & March, 2006). Although both fear learning and extinction change over the course of childhood (Glenn, Klein, et al., 2012; Kim & Richardson, 2010), there appears to be a marked change in these fear processes during adolescence (see Baker, Den, Graham, & Richardson, 2014 for review). Animal models have shown that adolescent rats have both enhanced fear acquisition (Hefner & Holmes, 2007) and reduced extinction learning (Pattwell, Lee, & Casey, 2013) and retention (McCallum, Kim, & Richardson, 2010) relative to juvenile and adult rats. Similarly, studies in human adolescents demonstrate reduced fear extinction (Pattwell et al., 2012) and overgeneralization of fear to safety cues (Lau et al., 2011). These findings suggest that adolescence is characterized by an increase in both fear learning and generalization—and a decrease in fear extinction—a combination that may contribute to the increased vulnerability to anxiety disorders during this developmental period.

Fear extinction shares many features with the more basic, but less often investigated, process of habituation. Although conceptually similar, there are critical differences between fear extinction and habituation. Fear extinction is the reduction in fear response that occurs with the repeated presentation of a conditioned stimulus (CS) in the absence of a previously-paired aversive unconditioned stimulus (UCS). As such, fear extinction involves the ability to learn alternative representations of previously feared stimuli (Jovanovic et al., 2014; Quirk, 2006). Alternatively, habituation is the reduction of a response following repeated presentation of the same stimulus—a decrement in response that is associated with the reduced salience of repeatedly presented stimuli (Groves & Thompson, 1970; Abel, Waikar, Pedro, Hemsley, & Geyer, 1998). Even the response to aversive or fear-eliciting stimuli habituate over time, and this is observed across multiple measures (e.g., startle response, galvanic skin response, neural activity in the amygdala).

Similar to aberrations in fear learning and extinction, *reduced habituation* of physiological response to aversive stimuli may reflect an underlying mechanism involved in vulnerability for anxiety disorders. Reduced habituation of physiological responses has been associated with greater anxiety (Lader & Wing, 1964; Ludewig et al., 2005), anxiety sensitivity (Campbell et al., 2014), neuroticism (Norris, Larsen & Cacioppo, 2007), and panic attacks (Roth, Ehlers, Taylor, Margraf, & Agras, 1990). Similarly, reduced habituation was associated with greater anxiety symptoms in adolescence (Hare et al., 2008) and has been observed in children at increased risk for developing an anxiety disorder based on current parental anxiety disorder status (Turner, Beidel, & Roberson-Nay, 2005). In one of few studies to examine the effect of sex on habituation in children, Thomas et al. (2001) demonstrated that left amygdala activation habituated to fearful faces among 8–13-year-old children—but only in boys. Hence, reduced habituation to fearful stimuli was unique to adolescent girls, who are at increased risk for anxiety relative to boys (Lewinsohn, Gotlib, Lewinsohn, Seeley, & Allen, 1998).

Taken together, these findings suggest that adolescence is a critical developmental period when aberrations in habituation may emerge. The transition from childhood into this vulnerable period is marked by *puberty*, a series of physical, neural and hormonal changes associated with the progression into adolescence (Casey et al., 2008; Spear, 2000). Pubertal development has been directly associated both with changes in fear processes (Quevedo, Benning, Gunnar, & Dahl, 2009; Schmitz et al., 2014; Spielberg, Olino, Forbes, & Dahl, 2014), and with an increased vulnerability for anxiety (Graber, Lewinsohn, Seeley, & Brooks-Gunn, 1997; Reardon, Leen-Feldner & Hayward, 2009). Furthermore, pubertal development has been shown to have a greater impact on fear-related neural systems in girls relative to boys (Bramen et al., 2011).

In addition to typical pubertal development, a number of other measures have been shown to relate to risk for anxiety, including self-reported behavioral inhibition system, and the error-related negativity (ERN). The Behavioral Inhibition System is a part of the BIS/BAS framework originally proposed by Gray (1987) to conceptualize the systems that motivate adaptive approach and avoidance behaviors (Carver & White, 1994). The BIS is the motivational system that drives inhibition and withdrawal behaviors, attention to threat, and increased arousal in the face of threat, punishment, and novelty. Elevated BIS sensitivity is a vulnerability factor for the development of an anxiety disorder (Johnson, Turner & Iwata, 2003), is associated with anxiety symptoms and negative affect more generally (Amodio, Master, Yee, & Taylor, 2008; Boksem, Tops, Weseter, Meijman & Lorist, 2006; Carver & White, 1994), and has been associated with an enhanced startle response (Caseras et al., 2006). Furthermore, greater behavioral inhibition—a temperamental disposition similarly associated with sensitivity to threat, novelty, and risk for anxiety—has been characterized by reduced habituation of amygdala and hippocampal activation to neutral and novel faces (Blackford, Avery, Cowan, Shelton, & Zald, 2011; Blackford, Allen, Cowan, & Avery, 2013). Together, these findings suggest that greater trait BIS sensitivity is associated with enhanced and sustained fear responding and an increased risk for anxiety.

A growing body of evidence has found that risk for anxiety is also associated with an increased neural response to errors (i.e., a larger error-related negativity, ERN; Moser et al., 2013; Cavanaugh & Shackman, 2015). Errors are unpredictable threatening events that are experienced as aversive (Botvinick, 2007; Dreisbach & Fischer, 2012) and trigger defensive responding such as increased eye blink startle response (Hajcak & Foti, 2008; Riesel et al., 2013), corrugator muscle activation (Lindstrom et al., 2013), skin conductance response (Hajcak et al., 2003, 2004), and amygdala activation (Pourtois et al., 2010). The ERN is an electrophysiological index of error processing that is observed as a negative deflection in the event-related potential that peaks within 100 ms following the commission of an error (Gehring et al., 1993; Hajcak, 2012; Meyer, Weinberg, Klein, & Hajcak, 2012). The ERN is observed across the lifespan and is measurable in children as early as 3–5 years of age (Grammer et al., 2014; Torpey et al., 2009). Similar to BIS sensitivity—individual differences in the ERN are posited to index trait-like threat sensitivity, with a larger ERN being associated with greater sensitivity to potential threat and risk for anxiety (Weinberg, Dieterich, & Riesel, 2015; Proudfit, Inzlicht, & Mennin, 2013). Consistent with this notion, research has shown that the ERN is enhanced in individuals with greater BIS sensitivity, harm avoidance, and neuroticism (Amodio et al., 2008; Boksem et al., 2006). Moreover, the ERN is larger in individuals with generalized anxiety disorder (Weinberg et al., 2010, 2012), obsessive compulsive disorder, (Endrass et al., 2008; Gehring & Knight, 2000; Hajcak, Franklin, Foa, & Simons, 2008; Xiao et al., 2011), and elevated worry (Hajcak et al., 2003).

The association of the ERN with anxiety is also observed in children and adolescents. Indeed, the ERN is larger in children with anxiety disorders (Ladouceur et al., 2006; Meyer et al., 2012, 2013; Hajcak et al., 2008) and a larger ERN predicts the onset of new anxiety disorders in children (Meyer et al., 2015). Furthermore, previous studies have demonstrated that the relationship between early behavioral inhibition and later clinical disorders is moderated by the magnitude of the ERN (McDermott et al., 2009; Lahat et al., 2014). Thus, both BIS sensitivity and the ERN appear to reliably index anxiety and risk for anxiety across the lifespan, with higher BIS scores and a larger ERN repeatedly associated with greater risk for anxiety disorders.

The present study examined the unique associations between pubertal development and two measures of risk for anxiety (BIS and the ERN), with startle habituation during a fear-learning paradigm in 8–14-year-old girls. Participants completed two primary tasks: 1) a speeded reaction time task (i.e., an arrow version of the Flanker task, Eriksen & Eriksen, 1974) in which subjects occasionally make errors—used to elicit the ERN, and 2) a screaming faces version of a fear learning paradigm used in previous research in children and adolescents (Lau et al., 2008; Glenn, Klein, et al., 2012). In the flanker task, participants were shown five arrowheads, and instructed to press the left or right mouse button—as quickly and accurately as possible—to indicate which direction the center arrow was pointing. EEG data are collected as participants complete the task, from which we are able to measure the magnitude of the ERN. Second, participants completed the fear learning paradigm. During this task participants were shown images of two neutral female faces: one serves as the conditioned threat cue (CS+) and the other as the conditioned safety cue (CS−). As an unconditioned stimulus, the CS+ female face changes to a fearful expression concurrent with the presentation of a loud scream (Glenn, Klein, et al., 2012; Glenn, Lieberman, & Hajcak, 2012; Lau et al., 2008; Lau et al., 2011). In this task, the startle reflex was elicited during the CS+ and CS− trials, and both fear-potentiated startle (FPS; i.e., the difference between CS+ and CS−) and startle habituation were examined across the task. FPS is the increased startle response observed when an organism is in a negative relative to positive or neutral state, and is one of the most reliable measures of fear learning (Glenn, Klein, et al., 2012; Lang, Davis & Ohman, 2000).

In previous studies, startle habituation was assessed by (1) calculating the difference between the last and first startle response (Ellwanger, Geyer, & Braff, 2003), (2) measuring the number of trials before a subthreshold response was reached (Turner et al., 2005), or (3) calculating the change in startle response across blocks of trials (Liberman et al., 2006). Although these approaches index habituation, they do not take into account rates of change and do not incorporate individual trial magnitudes. In the current study, a mixed linear model approach was utilized to generate a unique habituation slope for each participant, allowing for a more fine-grained examination of startle habituation over time (Campbell et al., 2014; LaRowe, Patrick, Curtin, & Kline, 2006).

Overall then, the present study examined the impact of pubertal development and two measures that have been linked to anxiety risk—specifically self-reported BIS and ERN—on startle habituation during a fear-learning paradigm in adolescent girls. The study had three central hypotheses. First, based on the aberrant fear extinction and habituation associated with pubertal development (Hare et al., 2008; Pattwell et al., 2012; Thomas et al., 2001), we hypothesized that the startle response would habituate over time, and that increased pubertal development would be associated with *decreased* habituation to conditioned threat cues (i.e., the CS+). Second, we hypothesized that higher BIS would be associated with a larger (i.e., more negative) ERN. Third, given previous work demonstrating a relationship between risk for anxiety and both BIS and ERN (Johnson et al., 2003; Meyer et al., 2012; Meyer et al., 2013), we hypothesized that high BIS and large ERN would be associated with *decreased* startle habituation to conditioned threat cues (i.e., the CS+). In exploratory analyses we examined whether the associations between pubertal development, BIS, and the ERN and startle habituation were independent of each other.

2 | METHOD

2.1 | Participants

Participants were 77 girls recruited from the community as part of a longitudinal study on the impact of puberty on affect and neural development in adolescence. Given the increased vulnerability for anxiety and depression observed in young girls relative to boys, the study focused specifically on adolescent females. Families were recruited from the suburban New York area via a commercial mailing list, referrals, and other advertisements. Of 77 girls who started the task, 11 girls stopped before completion, either due to fear of the task ($n = 8$), or because of technical difficulties ($n = 3$). This drop-out rate is slightly higher than other fear conditioning studies, but is consistent with previous studies that have used the screaming faces paradigm in this age range (Glenn, Klein, et al., 2012; see description below). Participants were also excluded for having excessive artifacts in the baseline period (e.g., 50 ms before the startle probe; $n = 5$), or having no measurable startle response on more than 2/3 of startle trials in any one condition ($n = 7$). The final sample consisted of 54 girls ranging from 8 to 15 years old ($M = 13.20$ years, SD = 1.56). The sample was 83% Caucasian, 9% African American, and 7% Hispanic. The final sample did not significantly differ from excluded children on demographics, BIS, or ERN ($p > 0.2$). Furthermore, the children who stopped before completion due to fear of the task ($n = 8$) did not significantly differ from included participants on Age, pubertal development, BIS, or ERN ($ps > 0.10$). The study was approved by the Institutional Review Board of Stony Brook University, and all parents and children consented/assented to participation in the study.

2.2 | Measures

2.2.1 | Pubertal development

To measure pubertal development, participants completed the Puberty Development Scale (PDS; Petersen, Crockett, Richards, & Boxer, 1988; Carskadon & Acebo, 1993). The PDS consisted of five items that assessed growth spurt (e.g., increased height), breast and body hair development, skin changes (e.g., skin irritation or pimples), and the onset of menstruation. Development is rated across five items using a 4-point Likert scale ranging from 1 (*development has not yet started)* to 4 (*development seems complete*). The PDS has good psychometric properties and reliably measures pubertal development (Petersen, Crockett, Richards & Boxer, 1988). Pubertal development was scored and analyzed as the average PDS score as reported by the participant, with higher values indicating greater pubertal development.

2.2.2 | BIS sensitivity

To measure BIS sensitivity, participants completed the BIS/BAS questionnaire. The BIS/BAS-scale is a 24-item measure frequently utilized in psychological research to assess dispositional sensitivities to reward and threat. The present analyses focused on the BIS-scale as an index of individual differences in concern over the possible occurrence of negative events, and sensitivity to negative events when they do occur (Carver & White, 1994; Jorm et al., 1998). The 7-item BIS subscale has been shown to correlate with broad trait measures of threat sensitivity, including trait anxiety, negative affectivity, negative temperament, and harm avoidance (Boksem et al., 2006; Carver & White, 1994), and has been used in child and adolescent samples (Cooper et al., 2007).

2.3 | Stimuli

The screaming faces version of the fear learning task was based on a similar version utilized in previous studies (see Figure 1) (Glenn, Klein, et al., 2012; Lau et al., 2008). Participants were presented with two

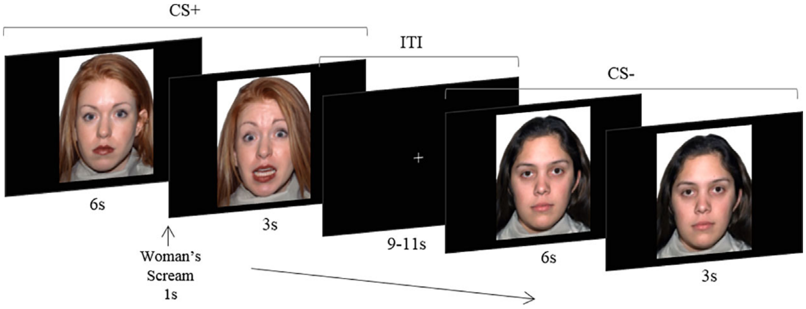

FIGURE 1 Schematic of the screaming faces paradigm. In the CS+ condition participants were presented with the image of a woman's neutral face for 6s, followed by a fearful screaming face for 3s concurrent with a woman's scream for 1s. The CS− trials consisted of the presentation of a neutral face for 9s. The inter-trial interval (ITI) consisted of the presentation of a white fixation cross on a black screen for 9–11s

neutral female faces from the NimStim stimulus set (01F and 03F; Tottenham et al., 2009). One image served as the CS+ and the other as the CS−, counterbalanced across participants. The neutral CS+ face was first presented for 6s. The UCS was an image of the CS+ actress with a fearful expression presented for 3s, concurrent with a loud female scream presented through speakers that lasted approximately 1s. Thus, on reinforced trials the neutral CS+ face appeared to suddenly look fearful and scream (i.e., UCS). The CS+ was reinforced with the UCS on 75% of trials. The CS− trials consisted of the presentation of a neutral face for 9s and were never paired with the UCS.

2.4 | Procedure

Participants completed a lab visit that lasted approximately 4–5 hr and consisted of multiple tasks, including diagnostic interviews, self-report measures, and psychophysiological and neuroimaging tasks. Additional data are presented in other reports (Ferri et al., 2014; Speed, Nelson, Auerbach, Klein, & Hajcak, 2016). Relevant to the current study, participants completed a flanker task while continuous EEG data were collected to measure the ERN, and a fear learning startle paradigm while startle eye blink response was measured. All participants completed the flanker task before the startle task. Participants and parents were informed that the startle task involved the presentation of fear-provoking and neutral stimuli. As such, before beginning the task, participants were reminded that they could discontinue participation at any time.

2.4.1 | Flanker task

During EEG data collection, participants completed an arrow version of the flanker task. In this task, children were seated approximately 24 in from a computer screen, while they performed an arrow version of the flanker task (Eriksen & Eriksen, 1974). The task was administered using Presentation software (Neurobehavioral Systems, Inc., Albancy, CA) to control the presentation and timing of all stimuli. On each trial, five horizontally aligned arrowheads were presented for 200 ms, followed by an ITI that varied between 2300 and 2800 ms. Half of the trials were compatible ("<<<<<" or ">>>>>") and half were incompatible ("<<><<" or ">><>>"); the order of trials was randomly determined. Participants were instructed to respond as quickly and as accurately as possible by pressing the right mouse button if the center arrow was pointing to the right, and the left mouse button if the center arrow was pointing left. Participants then completed a practice block of 30 trials to ensure adequate performance, followed by the full task which consisted of 11 blocks of 30 trials (330 trials total); each block was initiated by the participant. At the end of each block, participants received feedback based on their performance. If performance was 75% correct or lower, the message "Please try to be more accurate" was presented; if performance was above 90% correct, the message "Please try to respond faster" was displayed; otherwise the message "You're doing a great job" was shown.

2.4.2 | Fear-learning task

During the fear-learning task, participants were seated in front of a 13-inch computer monitor in a sound-attenuated and dimly lit booth. In the chamber, headphones were placed over their ears, and electrodes were positioned below the left eye and on the forehead. The task began with a baseline phase included to reduce extreme startle response in the first few trials. During the initial baseline phase, participants were presented with four neutral or fearful female faces from the NimStim stimulus set (05F Neutral, 09F Fearful, 11F Neutral and 18F Neutral; Tottenham et al., 2009) and the aversive female scream. None of the faces were the same as the CS+ or CS− faces. Startle probes were presented through headphones as participants viewed the faces.

Following the baseline phase, participants were told that they would see pictures of two different women, and they were instructed to watch the pictures as they appeared on the screen. Participants were further informed that one of the women may change to look afraid, and then let out a scream through the computer speakers. Participants were told that they may be able to predict when the fearful scream would occur if they paid close attention. Lastly, participants were told they would continue to hear the loud sound (i.e., startle probe) through the headphones occasionally, but to ignore those sounds and focus on the pictures. Participants then completed the task, which lasted approximately 10 min.

The task consisted of 16 total trials: 8 CS+ and 8 CS−. Across conditions, the startle probe was presented on 75% of trials, resulting in six startle trials for both the CS+ and CS−. The inter-trial interval (ITI) consisted of the presentation of a white fixation cross on a black screen for 9–11s. Four ITI startle probes were presented to reduce predictability of the startle probe. In total there were 6 CS+, 6 CS−, and four ITI startle trials; presentation of the CS+ and CS− trials were randomized across the task; 75% of CS+ trials were reinforced with a UCS—and the first CS+ trial was always reinforced.

Following completion of the startle task, participants were instructed to rate how afraid they felt when they saw the CS+ and CS− stimuli. Participants were given a 5-point Likert scale ranging from 0 (*not afraid at all*) to 4 (*very afraid or nervous*). In order to assess contingency awareness, participants were also asked to indicate which face they believed was followed by the scream.

2.4.3 | Physiological data recording, reduction, and analysis

EEG

Continuous EEG was recorded during the flanker task using an elastic cap with 34 electrode sites placed according to the 10/20 system and two electrodes on the left and right mastoid. Electrooculogram was recorded from electrodes placed above and below the right eye and two placed on the outer canthus of both eyes. All electrodes were sintered Ag/AgCl electrodes. Data were recorded using Active Two BioSemi system (BioSemi, Amsterdam, Netherlands). The EEG was digitized with a sampling rate of 1024 Hz using a low-pass fifth order sinc filter with a half-power cutoff of 102.4 Hz. A common mode sense active electrode producing a monopolar (non-differential) channel was used as recording reference. EEG data were analyzed using Brain Vision Analyzer (Brain Products, Gilching, Germany).

Data were referenced offline to averaged mastoids, band-pass filtered with low and high cutoffs (0.1 and 30 Hz, respectively), and corrected for eye movement artifacts (Gratton, Coles, & Donchin, 1983). Response-locked epochs with duration of 1500 ms, including a 500 ms pre-response interval, were extracted. Epochs containing a voltage greater than 50 µV between sample points, a voltage difference of 300 µV within a segment, or a maximum voltage difference of less than 0.50 µV within 100 ms intervals were rejected. Additional artifacts were identified and removed based on visual inspection. The 500–300 ms pre-response interval was used as the baseline (Weinberg et al., 2010).

A negative deflection is observable after both error (i.e., the error-related negativity, ERN) and correct trials (i.e., correct response negativity, CRN). In addition to the CRN and ERN, we also calculated their difference (ERN minus CRN; ΔERN) to isolate activity unique to error processing (Simons, 2010). The ERN and CRN were quantified as the mean amplitude between 0 and 100 ms after responses at electrode FCz, where the ΔERN was maximal.

Startle

Startle-elicited EMG activity was collected in accordance with current guidelines outlined by Blumenthal et al. (2005). Two electrodes, 4 mm diameter Ag/AgCl filled with electrode gel (TD-40; Mansfield R and D), were positioned beneath the left eye over the orbicularis oculi muscle approximately 25 mm apart. A third electrode was placed on the forehead to serve as an isolated ground. EMG activity was recorded using a PSYLAB Stand Alone Monitor (SAM) unit and an attached BioAmplifier system (Contact Precision Instruments; Cambridge, MA). EMG activity was sampled at 1,000 Hz and filtered between 30 and 500 Hz. EMG responses were rectified in a 200 ms window, beginning 50 ms before the onset of the startle probe. A 6-point running average was applied to the rectified data to smooth out sharp peaks. Raw startle magnitude was baseline corrected and represented the difference between the average of the EMG in the 50 ms window prior to the startle probe and the maximum in the 150 ms post-probe window. Each participant's data were examined on a trial-by-trial basis. Trials with no perceptible eye-blink response (i.e., a blink response approximately 10 mV or less) were scored as zero and included in the overall averages; trials with excessive baseline artifacts or noise were excluded from analysis. Non-responders were participants who had visible startle response on fewer than 3 (50%) of usable trials in any one condition; non-responders were excluded from analyses.

2.5 | Statistical analysis

Self-reported anxiety ratings during the presentation of the CS+ and CS− were compared using a paired-samples *t*-test. A repeated measures analysis of variance (ANOVA) was used to evaluate the mean startle response elicited during the CS+, CS−, and ITI. Bonferroni correction was applied to all ANOVA post hoc *p* values to adjust for multiple pairwise comparisons.

To assess the association of self-reported and neural measures of pubertal development and threat sensitivity, we conducted zero-order correlations of the PDS, BIS, ΔERN, and mean startle measures (i.e. CS +, CS−, ITI, FPS). To investigate the change of startle magnitude over the course of the task (i.e., startle habituation), we conducted a mixed linear model, which included startle magnitude as a dependent variable, and tested for main effects of trial (i.e., habituation), condition (CS+ vs. CS−), puberty (PDS), BIS, and ΔERN magnitude; all continuous variables were mean centered.

Three primary analyses were conducted to examine startle habituation. The first analysis tested the association between pubertal development (PDS) and startle habituation. The model included the main effect of trial, condition, PDS, and the Trial X Condition, Trial X PDS, and Trial X Condition X PDS interaction terms. A second analysis was conducted to examine the association of startle habituation with BIS—the model included the main effects of trial, condition, BIS, and the Trial X Condition, Trial X BIS, and Trial X Condition X BIS interaction terms. Lastly, a third analysis was conducted to examine the association of error-related brain activity with startle habituation—the model included main effects of trial, condition, ΔERN, and the Trial X Condition, Trial X ΔERN, and Trial X Condition X ΔERN interaction terms. Significant interactions were followed-up by examining startle habituation at ±1 SD of the moderators (Campbell et al., 2014; Holmbeck, 2002). Furthermore, in order to assess for the independent contribution of puberty, BIS, and the ΔERN in predicting startle habituation, Age, PDS, ΔERN, and BIS values were included as covariates in each analysis. All effect sizes are provided as partial eta-squared (η_p^2) for *F* tests and Cohen's *d* for *t* tests.

3 | RESULTS

3.1 | Self-report ratings and contingency awareness

A within-subjects *t*-test of self-reported anxiety during the task revealed that participants reported more fear during the CS+ ($M = 1.38$, $SD = 1.03$) relative to the CS− ($M = 0.62$, $SD = 0.84$), $t(53) = 5.15$, $p < .001$, $d = 0.81$. Previous research has demonstrated that adolescents who correctly learn the UCS-CS+ pairing show fear response patterns that differ from those participants who do not learn the UCS-CS+ association (Glenn, Klein, et al., 2012). Given this, we examined the effect of contingency awareness on mean startle response patterns. Assessment of contingency awareness showed that 47 participants were *learners* (i.e., correctly identified the CS+ face) and 7 were *non-learners* (i.e., incorrectly identified the CS− face). Learners ($M = 13.43$ years, $SD = 1.29$) were significantly older than non-learners ($M = 11.64$ years, $SD = 2.34$), $t(52) = -3.05$, $p < .01$, $d = 0.95$. There was a trend towards a difference in pubertal development, such that learners ($M = 2.91$, $SD = 0.81$) were slightly more advanced in pubertal development than non-learners ($M = 2.31$, $SD = 1.01$), $t(52) = -1.75$, $p < 0.10$, $d = 0.66$. However, a one-way analysis of covariance showed that the trending difference of pubertal development across learning groups was no longer trending significance after controlling for the effect of Age, $F(1,51) = .41$, *n.s.* A 2 (Learners vs. Non-Learners) × 3 (CS+, CS−, ITI) mixed-model ANOVA showed that learners (CS+: $M = 63.82$, $SD = 22.18$; CS−: $M = 61.23$, $SD = 23.21$; ITI: $M = 56.07$, $SD = 22.86$) did not significantly differ from non-learners (CS+: $M = 74.20$, $SD = 20.73$; CS−: $M = 63.73$, $SD = 20.04$; ITI: $M = 62.30$, $SD = 24.21$) in magnitude of startle response to the CS+, CS−, or ITI, $F(1, 52) = 0.54$, $p > 0.45$. As such, non-learners were included with learners in all analyses; however, learner status was included as a covariate in all mixed linear model analyses to control for the possible impact of learning status on startle habituation.

3.2 | Fear conditioning

As shown in Figure 2A, mean startle magnitude during the CS+ ($M = 65.17$, $SD = 22.09$), CS− ($M = 61.55$, $SD = 22.66$), and ITI ($M = 56.88$, $SD = 22.90$) significantly differed from one another, $F(2, 106) = 11.39$, $p < .001$, $\eta_p^2 = 0.18$. Bonferroni post-hoc analyses confirmed that the startle response during the CS+ was significantly larger relative to the CS−, $p < .05$, $\eta_p^2 = 0.11$, and ITI, $p < .001$, $\eta_p^2 = 0.27$, and significantly larger during the CS− relative to the ITI, $p < .05$, $\eta_p^2 = 0.11$. These results remained significant after including pubertal development as a covariate (*ps* <.05).

3.3 | Correlations of self-report, psychophysiological, and neural measures

Table 1 displays Pearson's correlation between self-report, psychophysiological, and neural measures. There were no significant zero-order correlations observed between mean startle magnitude or FPS and age, puberty, BIS, or the ΔERN (*ps* >0.30). Age and puberty, however, were highly correlated ($r = 0.75$, $p < .001$). Furthermore, a larger ΔERN was associated with higher self-reported BIS ($r = -0.31$, $p < .05$), and more advanced self-reported pubertal development ($r = -0.28$, $p < .05$).

3.4 | Puberty and startle habituation

Figure 2B shows the mean startle response for each CS+ and CS− trial. Mixed linear model analysis indicated a main effect of trial, $b = -3.09$, $t = -2.44$, $p < .05$, indicating startle habituation during the task. Analyses further revealed a significant Trial X Puberty interaction, $b = 3.06$, $t = 2.02$, $p < .05$; there were no significant Trial X Condition or Trial X Puberty X Condition interactions (*ps* > 0.45).[1] To follow up the significant Trial X Puberty interaction, puberty was recoded as two conditional predictors one SD above and below the mean (High and Low Puberty, respectively; Holmbeck, 2002; Campbell et al., 2014). We then conducted two mixed linear model analyses, one in high and one in low puberty groups. Analyses revealed a main effect of trial for

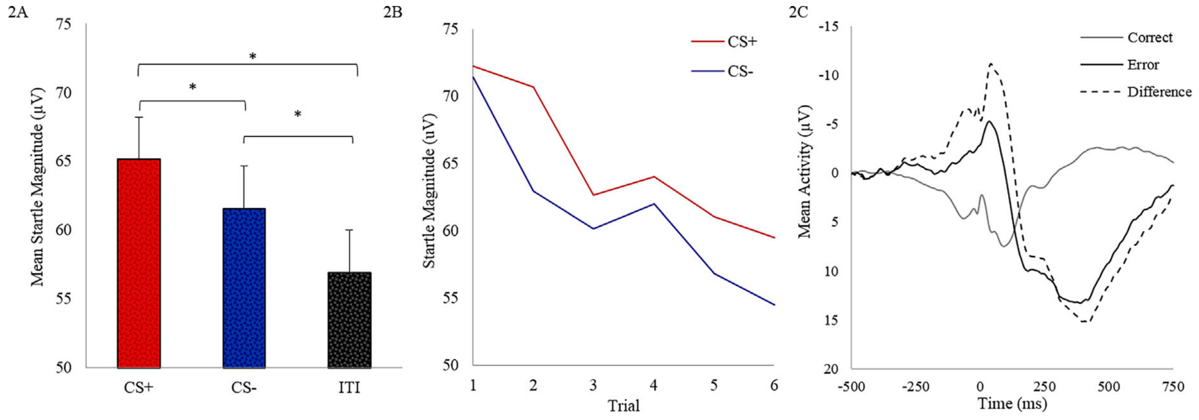

FIGURE 2 Panel A. Mean startle magnitude in µV in each condition. Means demonstrate startle to the CS+, CS−, and ITI. Error bars indicate the standard error of the mean, and startle magnitudes marked with an asterisk are significantly different from one another at $p < .05$. Panel B. Mean startle response for each trial in the CS+ (red line) and CS− (blue line). The pattern demonstrates a general habituation of startle response over the course of the task to both the CS+ and CS−. Panel C. Response-locked ERP waveforms for correct and error trials, along with the difference wave representing error minus correct trials

TABLE 1 Correlation of self-report, neural measures, and eye blink startle response

	M	SD	1	2	3	4	5	6	7
Self-report measures									
1. PDS	2.83	.85	—	—	—	—	—	—	
2. BIS	19.51	3.94	.20	—	—	—	—	—	
Neural measures									
3. ΔERN	−3.59	5.76	−.28*	−.31*	—	—	—	—	
Startle response									
4. CS+	65.17	22.09	−.12	−.01	−.02	—	—	—	
5. CS−	61.55	22.66	−.09	−.08	−.01	.89**	—	—	
6. ITI	56.88	22.90	.03	−.13	−.13	.81**	.82**	—	
7. FPS	3.62	10.56	−.06	.19	−.02	.18	−.29*	−.07	—

Note. M, mean; SD, standard deviation; BIS, behavioral inhibition system; PDS, puberty development scale; ΔERN, ERN minus CRN; CS+, conditioned threat cue; CS−, conditioned safety cue; ITI, inter-trial interval; FPS, fear-potentiated startle, CS+ minus CS−. Startle measures represent the mean magnitude of the startle response in each condition.
$^{*}p < .05$, $^{**}p < .01$.

low puberty, $b = -4.65$, $t = -6.30$, $p < .001$, but not for high puberty, $b = -1.10$, $t = -1.48$, $p > 0.15$, suggesting that only participants low in puberty habituated across trials. These effects were significant after controlling for age, learner status, BIS, and ΔERN, confirming that the effects were unique to pubertal development. Figure 3A shows mean values at each trial for high and low puberty groups. Estimated slopes for high and low puberty groups are shown in Figure 3B.

3.5 | BIS and startle habituation

Analysis revealed a main effect of trial, $b = -3.31$, $t = -2.60$, $p < .05$ and a significant Trial X BIS interaction $b = .92$, $t = 2.78$, $p < .01$; there was no significant Trial X Condition interaction ($p > 0.70$). The 2-way interaction was qualified by a significant three-way Trial X BIS X Condition interaction, $b = -0.38$, $t = -1.92$, $p = .05$. To follow-up this interaction, we evaluated rate of startle habituation in participants rated high and low on BIS separately in the CS+ and CS− conditions. In the CS+ condition, there was a main effect of trial in participants low in BIS, $b = -5.02$, $t = -5.05$, $p < .001$, but not high in BIS, $b = -0.95$, $t = -0.98$, $p > 0.30$ suggesting that participants low in BIS sensitivity showed expected habituation to the CS+, while those high in BIS did not habituate to the CS+ over time. Participants both high and low in BIS showed startle habituation during the CS− condition ($b = -0.38$, $t = -1.92$ and $b = -0.38$, $t = -1.92$, respectively, *ps* <.05). These effects remained significant after controlling for the effect of age, learner status, puberty, and the ΔERN. Figure 4A shows mean values at each trial for high and low BIS groups. The estimated slopes for high and low BIS groups are presented in Figure 5A.

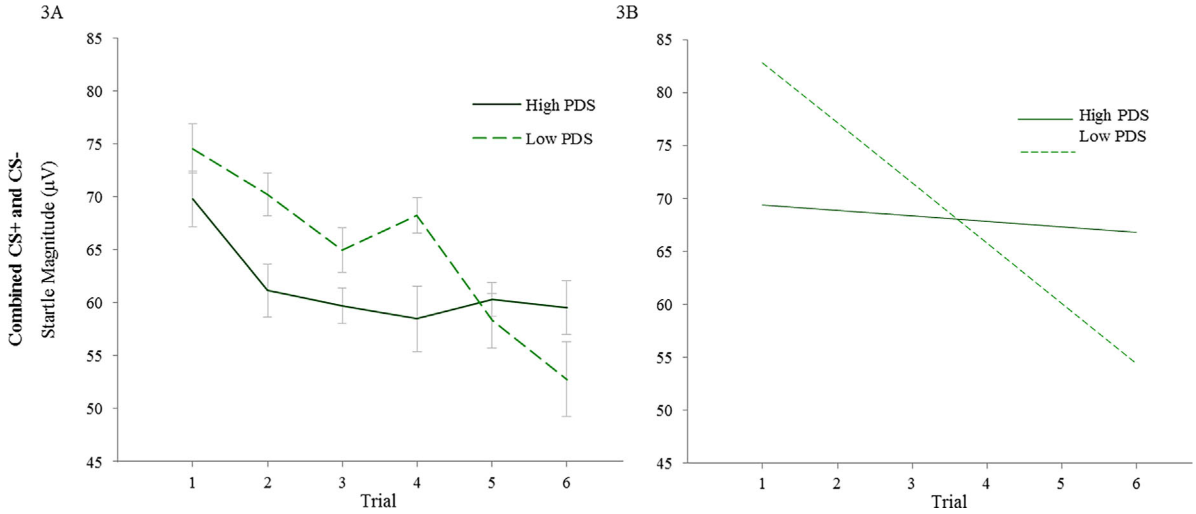

FIGURE 3 Panel A. Mean startle response for each trial modulated by self-reported pubertal development. High (solid line) and Low (dashed line) pubertal development groups were determined by median split. Mean startle for each trial is collapsed across the CS+ and CS−. Panel B. The modulation of estimated startle habituation slopes by pubertal development. Low self-reported pubertal development (dashed line) was associated with typical startle habituation across trials. Alternatively, more advanced pubertal development (solid line) was associated with a decrement in startle habituation across trials. Habituation presented is the overall habituation effect when collapsed across both the CS+ and CS−

3.6 | ERN and startle habituation

Figure 2C shows the average waveform of the CRN, ERN and ΔERN across all participants. Mixed linear model revealed a main effect of trial, $b = -3.26$, $t = -2.54$, $p < .05$ and a significant Trial X ΔERN interaction $b = -0.49$, $t = -2.20$, $p < .05$; there was no significant Trial X Condition interaction ($p > 0.70$). The 2-way interaction was qualified by a trending three-way Trial X Condition X ΔERN interaction, $b = -0.24$, $t = 1.80$, $p < 0.10$.[2] For consistency with BIS findings, we followed up this interaction by conducting two mixed linear model analyses in participants with large versus small ΔERNs in the CS+ and CS− conditions. Analyses in the CS+ condition revealed a significant effect of trial within small ΔERNs, $b = -4.45$, $t = -4.35$, $p < .001$, but no significant effect of trial within large ΔERNs, $b = -1.50$, $t = -1.50$, $p > 0.14$, suggesting that a larger ΔERN was associated with reduced habituation relative to smaller ΔERNs. Analyses in the CS− condition revealed startle habituation regardless of ΔERN magnitude, *ps* <.05. These effects remained significant after controlling for age, learner status, puberty, and BIS. Figure 4B shows mean values at each trial for high and low ΔERN groups, estimated slopes for high and low ΔERN groups are shown in Figure 5B.

3.7 | Startle intercepts

Finally, we examined whether there was an association between the intercept values of the startle response and PDS, BIS, or ΔERN. The intercept represents the magnitude of the startle response at the start of the task, as estimated by the value of the mixed linear model regression line at trial 1 (Shek & Ma, 2011). We conducted hierarchical linear regressions with 1) CS+ intercept, 2) CS− intercept, and 3) overall intercept (CS+ and CS− combined) as dependent variables. To control for age and contingency awareness, age and learner status were entered as independent variables in block 1, and PDS, BIS, and ΔERN were entered (in separate models) as independent variables in block 2. Age, PDS, BIS, and ΔERN were mean-centered continuous variables. Results indicated that there was no association between startle intercepts (CS+, CS−, or combined CS+/CS−) with PDS, BIS, or ΔERN (*ps* >0.25). These results suggest that neither pubertal development nor measures of threat sensitivity and anxiety risk (i.e., BIS and ERN) were associated with the initial startle response in either condition—although all were associated with habituation (i.e., slope) of the startle response across the task.

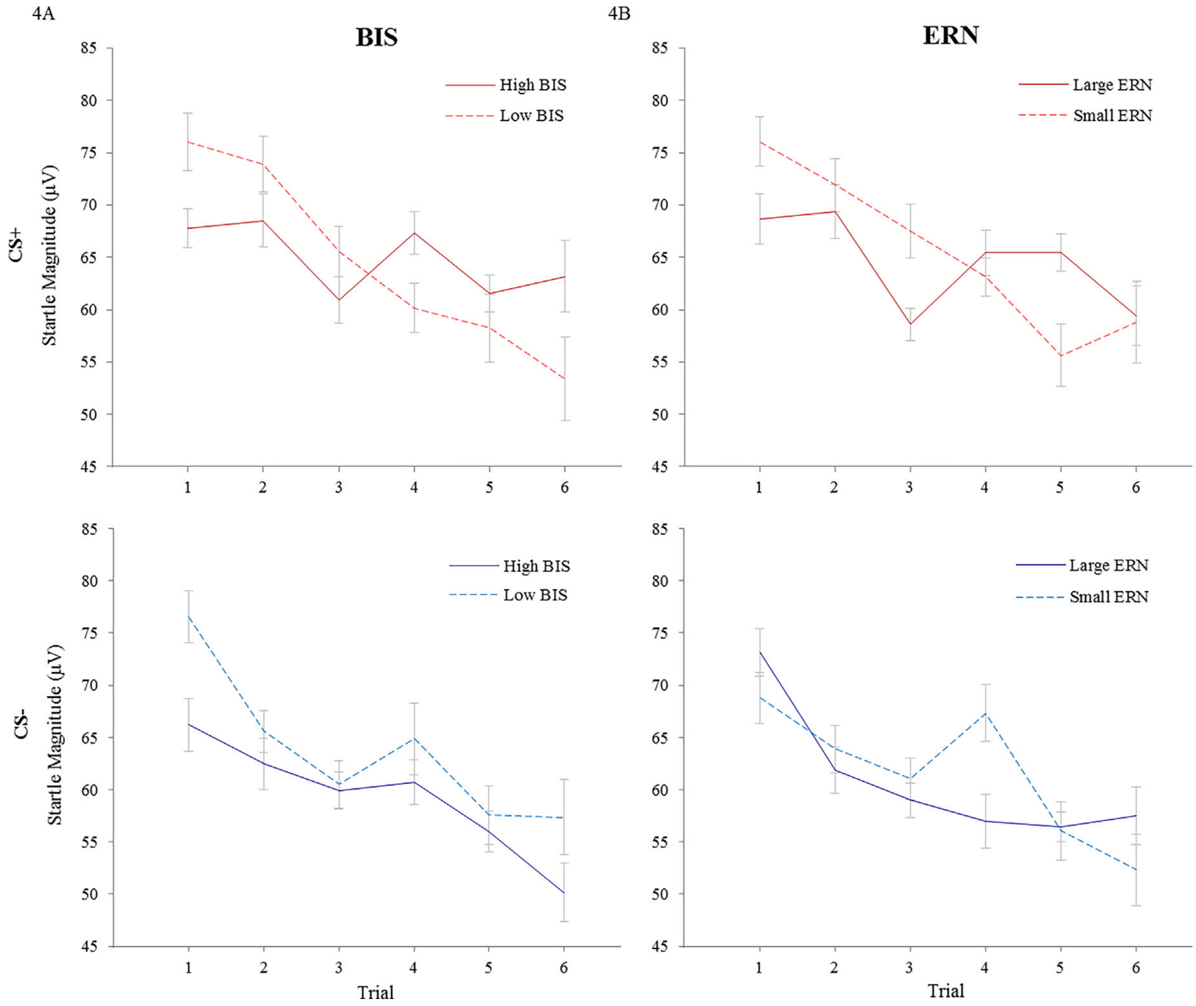

FIGURE 4 Mean startle response for each trial of the CS+ (top row) and CS- (bottom row) conditions modulated by self-reported BIS sensitivity and ΔERN magnitude. Panel A shows the modulation of trial by trial startle response by self-reported BIS. Panel B shows trial by trial mean startle response as modulated by ΔERN. High (solid lines) and Low (dashed lines) BIS and ΔERN shown in figure were determined by median splits

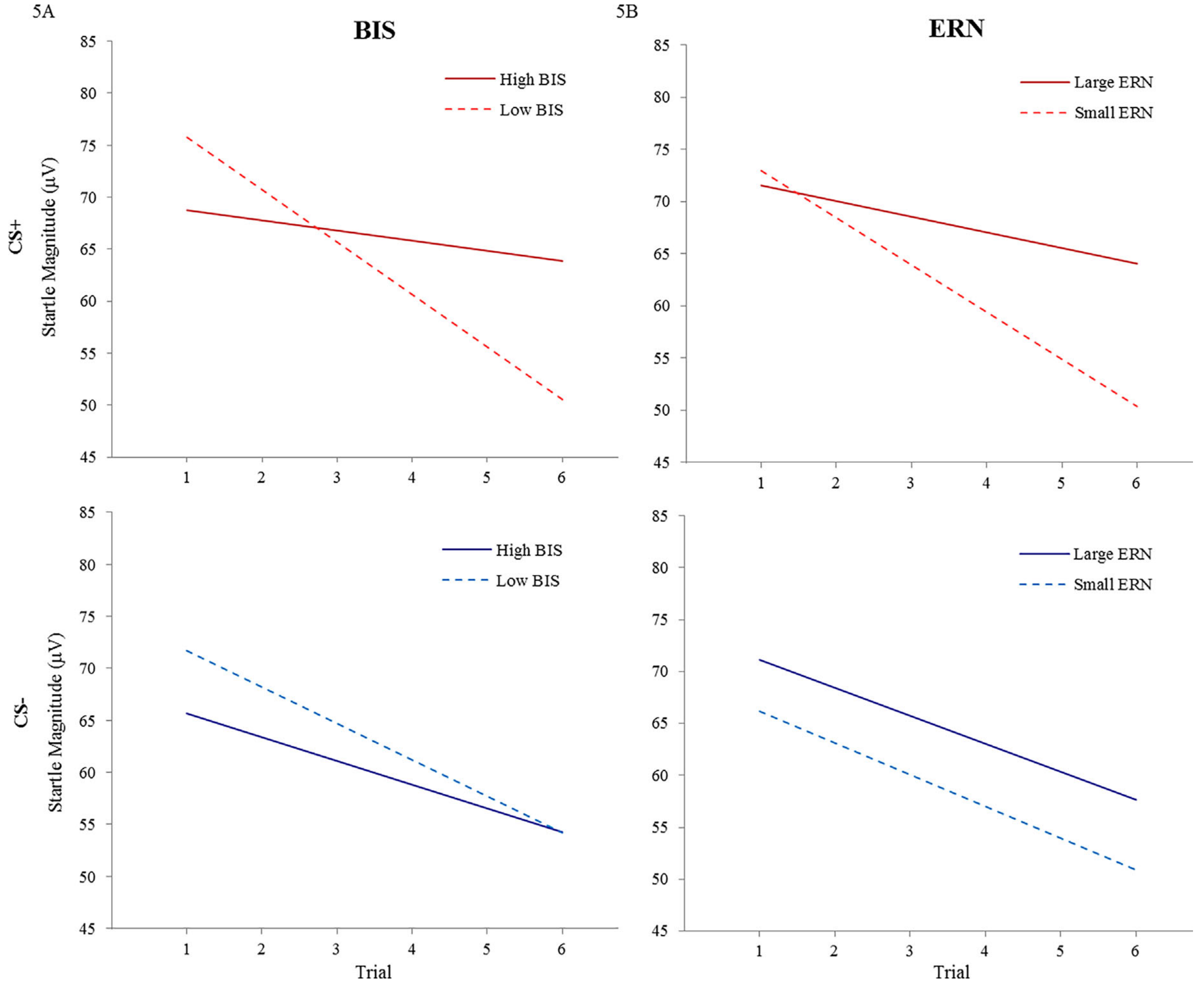

FIGURE 5 The modulation of estimated startle habituation slopes by self-reported BIS sensitivity and ΔERN magnitude in CS+ and CS− conditions. Panel A shows the modulation of estimated startle habituation slopes by self-reported BIS. Specifically, low BIS sensitivity was associated with typical startle habituation, while high BIS was associated with reduced startle habituation. This effect was observed in the CS+, but not CS−. Panel B shows that a large ΔERN was associated with failure to exhibit typical startle habituation, while a small ΔERN showed typical startle habituation across trials—this effect was observed in the CS+ but not the CS-

4 | DISCUSSION

The present study examined the impact of pubertal development and two measures that have been linked to anxiety risk (i.e., self-reported BIS and ERN) on startle habituation during a fear-learning paradigm in adolescent girls. Consistent with previous studies, we observed that the startle response to the CS+ was increased relative to CS− (i.e., FPS); however, mean startle response was not associated with pubertal development, BIS, or the ERN. In contrast, *habituation* of the startle response was associated with pubertal development and both BIS and ERN. Specifically, less advanced pubertal development, lower BIS sensitivity, and smaller ERNs were associated with typical habituation across the task. Alternatively, more advanced pubertal development was associated with reduced startle habitation to both conditioned threat and safety cues, while greater BIS and larger ERN were associated with reduced startle habituation specifically to conditioned threat cues. These effects were all independent of each other, and were not accounted for by the initial startle response.

Overall, the present study suggests that puberty is an important developmental process that impacts defensive habituation in adolescent girls in response to stimuli that signal both threat and safety. Measures directly related to risk for anxiety—greater BIS and larger ERN—were associated with reduced habituation *specifically* to the CS+. The current approach is consistent with the recent Research Domain Criteria (RDoC) initiative that emphasizes the importance of dimensional assessment of biological and self-report measures in the development and maintenance of psychopathology and risk (Cuthbert, 2014). By simultaneously examining puberty, BIS, and the ERN, we are able to demonstrate that these three measures account for unique variance in startle habituation.

Pubertal development and adolescence are associated with aberrant fear learning and extinction processes, potentially conferring increased risk for the development of anxiety disorders. Indeed, advancing puberty is shown to be associated with increased fear learning, decreased fear extinction, and increased development of anxiety disorders (Costello, Egger, & Angold, 2005; Graber et al., 1997; Reardon et al., 2009), with 6–18% of individuals reporting some form of anxiety during this period (Woodward & Ferguson, 2001). Given these vulnerabilities, there is a clear need to identify the maladaptive fear processes observed during this developmental period that may contribute to anxiety. The current findings are consistent with existent literature suggesting that aberrations in fear processes may be core vulnerabilities conferred with advancing puberty during adolescence. Specifically, the present findings demonstrate that defensive reflexes (i.e. startle response) habituate at a slower rate among adolescent girls with more advanced pubertal development—moreover, this effect was generalized to both the CS+ *and* CS− stimuli. These findings are consistent with the literature suggesting that failure to habituate and overgeneralization of the fear response to safety cues are associated with pathological anxiety (Ludewig et al., 2005; Lissek et al., 2005)—both of which appear to develop with typical advancing puberty. Relatively little research has been conducted to examine patterns of habituation during adolescence—therefore further research is necessary to better understand if these findings are the result of overgeneralization of the fear response or greater sustained processing of social stimuli in adolescence.

The present study replicates Glenn, Klein, et al. (2012) and demonstrates that adolescent girls exhibit typical FPS. However, Glenn, Klein, et al. (2012) also observed that older children have an increased FPS in the screaming faces task relative to younger children, presumably due to developmental changes in fear learning. In the present study, we did not observe any associations of mean startle response or FPS with age or puberty. These discrepant findings may be the result of key differences in the study samples. Although Glenn et al. sampled children from a similar age range (8–13 year-olds), their sample consisted primarily of boys (63%), whereas the current study sample was limited to adolescent girls. Given previous findings that highlight gender differences in the startle response (Schmitz et al., 2014), it is possible that conflicting findings are related to differences between boys and girls. Further research in larger samples is necessary to examine the potential role of gender differences in FPS and habituation.

Neither startle intercept nor FPS were directly associated with pubertal development or anxiety risk, suggesting that both the initial startle response and the potentiation of startle response to fearful stimuli did not differ as a result of advancing puberty or individual differences in threat sensitivity. These findings differ from research by Schmitz et al. (2014), who found that pubertal development was associated with increased FPS in adolescent girls. One possible explanation is that Schmitz et al. utilized geometric shapes as threat and safety cues, while the current study used fearful and neutral faces. Adolescents have increased sensitivity to social stimuli (Lau et al., 2008); therefore, these diverging results may be partially accounted for by differences between social and non-social stimuli. Indeed, children may process neutral or ambiguous stimuli as potentially threatening (Whalen, 1998), as indexed in previous studies by increased amygdala activation (Ferri et al., 2014; Thomas et al., 2001) and decreased amygdala habituation to neutral faces (Thomas et al., 2001).

In the current study, a larger ERN was associated with higher self-reported BIS. This finding is consistent with a growing literature supporting the association of ERN and individual differences in anxiety and threat sensitivity (Proudfit et al., 2013; Weinberg et al., 2015; Boksem et al., 2006). In addition to their zero-order correlation, BIS sensitivity and the ERN both demonstrated similar associations with startle habituation: higher BIS sensitivity and a larger ERN were both associated with reduced startle habituation on CS+ trials specifically. Each of these effects was observed even after controlling for variance accounted for by the other (i.e., ERN effects held after controlling for BIS). Given that these measures of threat sensitivity demonstrated very similar (although independent) interactive effects with startle habituation, the measures may together index a latent construct related to risk for anxiety. Data from the present sample were collected in the context of a longitudinal study, thus future examinations will prospectively investigate whether these measures (i.e., BIS, ERN, and startle habituation on CS+ trials) index unique or overlapping variance in risk for increased anxiety.

Although the mean startle response is a useful way of evaluating fear learning and extinction, the current results highlight the importance of evaluating startle responses at a more fine-grained, trial-by-trial level. Previous studies have shown that reduced startle habituation is observed both in individuals with greater anxiety sensitivity (Campbell et al., 2014) and those with anxiety disorders (Ludewig et al., 2005). Together, the results of this study demonstrate two distinct effects. First, our puberty findings demonstrate that advanced pubertal development is associated with reduced habituation to both threat and safety cues. These findings suggest that the changes in fear processes that occur during typical pubertal development are similar to those observed in anxiety. We are currently examining whether this pattern of reduced habituation predicts various psychopathology in later adolescence.

Alternatively, measures linked to greater risk for anxiety (i.e. higher BIS sensitivity, a larger ERN) were associated with failure to habituate *specifically* to CS+ stimuli, while typical habituation was observed to CS− stimuli regardless of BIS or ERN magnitude. As previously mentioned, meta-analytic examinations have demonstrated that anxiety is characterized not by an enhanced response to threat cues, but rather an enhanced fear response to safety cues, relative to healthy controls (Lissek et al., 2005). However, existent studies have largely examined *mean* startle response to threat and safety cues. The current findings suggest that aberrant fear responding to threat stimuli may indeed be an important aspect of anxiety—however the use of mean startle response in previous studies may have obscured these effects. These findings further suggest that, failure to habituate to conditioned threat cues may be an underlying mechanism that drives the vulnerability for anxiety associated with greater threat sensitivity as measured by high BIS sensitivity and a large ERN.

The present study is not without limitations. First, the sample was entirely female, limiting our ability to examine how these effects may differ across gender. Data collection took place in the context of a larger longitudinal study aimed to examine the effect of puberty on the development of internalizing disorders, and thus focused on adolescent females who are at elevated risk for such pathology. Future studies are needed to investigate how the effects of startle habituation —and its association with measures of risk for anxiety—may vary across gender. Second, the current sample size was small ($n = 54$), with a relatively high dropout rate ($n = 11$). Although dropout analyses suggest that there were no differences in pubertal development, BIS, or ERN between children who completed versus stopped the task, it will be important to replicate these findings in a larger sample. Larger samples will also provide important opportunities to replicate effects that were marginal, though predicted a priori (i.e., three-way interaction of startle habituation, ΔERN magnitude, and condition).

In sum, we found that startle habituation is related to pubertal development, BIS and the neural response to errors. Greater pubertal development was associated with failure to habituate to both conditioned threat and safety cues, suggesting that reduced habituation of the defensive fear response is observed with advancing puberty. Alternatively, measures linked to increased risk for anxiety—greater BIS sensitivity and a larger ERN—were associated with decreased habituation of the startle reflex specifically to conditioned threat stimuli. The similarities of these effects suggests that BIS, ERN, and habituation rates to CS+ may reflect a common latent variable related to risk for anxiety. Importantly, each of these effects was observed independently of the other, suggesting that each measure contributes to unique variance in habituation of defensive responding. These findings highlight the importance of examining responses on a trial-by-trial basis, and calls for future research examining latent variables of threat sensitivity, their association with fear habituation, and how they may prospectively predict anxiety.

ACKNOWLEDGMENTS

This study was funded by the National Institute of Mental Health grant R01 MH097767 awarded to Greg Hajcak. The authors would like to thank Emily Hale-Rude for her invaluable role in this project. Most of all, we would like to thank the children and families who participated in the iPANDA project, without whom this research would not be possible.

ENDNOTES

[1] Parallel analyses were conducted to investigate the impact of age on startle habituation. The mixed linear model analysis indicated a main effect of trial, $b = -3.17$, $t = -2.49$, $p < .05$, demonstrating overall startle habituation across the task. There was no main effect of condition, $b = -3.69$, $t = -1.26$, *ns*, age, $b = -2.31$, $t = -.60$, *ns*, or puberty, $b = -5.02$, $t = -1.05$, *ns*, and there were no significant interactions with age (*ps* >0.20).

[2] Parallel analyses were also conducted to examine the association of the CRN and ERN with startle habituation. Mixed linear model analyses with the CRN revealed no significant Trial X CRN ($b = -.31$, $t = 1.48$, *n.s.*) or three-way Trial X Condition X CRN ($b = -.16$, $t = -1.27$, *n.s.*) interactions. Similarly, mixed linear model analyses with the ERN showed no significant Trial X ERN ($b = -.09$, $t = -.47$, *n.s*) or Trial X Condition X ERN interactions ($b = -.03$, $t = -0.28$, *n.s*).

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How to cite this article: Jackson F, Nelson BD, Meyer A, Hajcak G. Pubertal development and anxiety risk independently relate to startle habituation during fear conditioning in 8–14 year-old females. *Developmental Psychobiolog*. 2017;59:436–448. https://doi.org/10.1002/dev.21506