NEURAL CORRELATES OF ANTICIPATION IN CHILDREN AT HIGH RISK FOR ANXIETY

By

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DEDICATION

To my family and friends, for their encouragement and support.

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LIST OF ABBREVIATIONS

Abbreviation	
ACC	anterior cingulate cortex
ADHD	attention deficit hyperactivity disorder
ADISC	Anxiety Disorders Interview Schedule for Children
ANOVA	analysis of variance
BIQ	Behavioral Inhibition Questionnaire
BIQ-C	Behavioral Inhibition Questionnaire – child version
BIQ-P	Behavioral Inhibition Questionnaire – parent version
BNST	bed nucleus of the stria terminalis
BOLD	blood oxygen-level-dependent
CSRI	Child Self-Report of Inhibition
CDI	Children's Depression Inventory
CRH	corticotropin-releasing hormone
dACC	dorsal anterior cingulate cortex
dlPFC	dorsolateral prefrontal cortex
dmPFC	dorsomedial prefrontal cortex
EPI	echo planar imaging
fMRI	functional magnetic resonance imaging
gPPI	generalized psychophysiological interaction toolbox
HPA	hypothalamic-pituitary adrenal

IQ	intelligence quotient
IT	inhibited temperament
KBIT	Kaufman Brief Intelligence Test
KSADS	Kiddie Schedule for Affective Disorders and Schizophrenia
MASC	Multidimensional Anxiety Scale for Children
mPFC	medial prefrontal cortex
MRI	magnetic resonance imaging
PFC	prefrontal cortex
PPI	psychophysiological interaction
rACC	rostral anterior cingulate cortex
RIBI	Retrospective Assessment of Behavioral Inhibition in Infants and Toddlers
ROIs	regions of interest
SCARED	Screen for Child Anxiety-Related Emotional Disorders
SCQ	Social Communication Questionnaire
SPAI-C	Social Phobia and Anxiety Inventory for Children
TMCQ	Temperament in Middle Childhood Questionnaire
UT	uninhibited temperament

Chapter I

INTRODUCTION

Conceptual Framework

Social Anxiety

Social anxiety disorder is common, disabling, and develops early in life. As the third most common psychiatric disorder, social anxiety disorder affects about 15 million Americans annually (Kessler et al., 2005), and prevalence rates are increasing (Heimberg et al., 2000). Symptoms of social anxiety disorder include the persistent fear of negative evaluation by others, such as being embarrassed, humiliated, or rejected (Heimberg et al., 2014). Almost seventy percent of patients with social anxiety disorder will develop a co-morbid psychiatric disorder (Schneier et al., 1992)and the disorder is associated with substantial disability (Katzelnick et al., 2001). Finally, social anxiety disorder has an early onset, resulting in a long duration of illness; 50% of patients develop social anxiety disorder by age 13 and 75% of patients have the disorder by age 15 (Kessler et al., 2005).

Social anxiety disorder follows a developmental trajectory (Ollendick and Hirshfeld-Becker, 2002). Children with social anxiety disorder avoid unfamiliar peers and adults, and may remain on the outskirts of a group of children, watching, rather than joining in the play. Children with social anxiety disorder

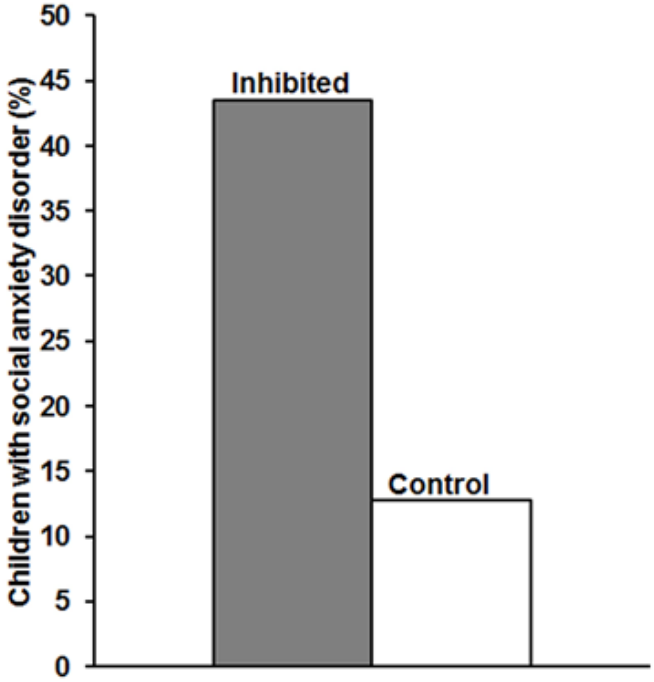
may not be able to express their fears of social-evaluative situations, but instead throw a tantrum or refuse to talk to unfamiliar people. These symptoms can prevent children from learning age-appropriate social interactions, resulting in more social anxiety and impairment, and may prevent the child from participating in school, resulting in lower academic achievement. As children grow older, they develop an awareness of themselves and how others perceive them. Children in middle to late childhood begin to compare themselves to others and to think about consequences of their actions. Increasing awareness of social situations and social consequences may lead to anticipatory anxiety, a critical process in the development of social anxiety symptoms (Morris et al., 2005). During adolescence, peer relationships become increasingly important and adolescents with social anxiety may be excluded by their peers (Blumenthal et al., 2009). The combination of peer exclusion and increasing sensitivity to embarrassment leads to the sharp uptick in social anxiety diagnoses during adolescence.

Inhibited Temperament

Social anxiety disorder is often preceded by inhibited temperament (Chronis-Tuscano et al., 2009; Clauss and Blackford, 2012; Essex et al., 2010; Schwartz et al., 1999), one of the most studied behavioral phenotypes. Pioneering research by Jerome Kagan (Kagan and Moss, 1962) established inhibited temperament as a fundamental behavioral response to unfamiliar events. Inhibited temperament can be observed across cultures (Meysamie et

al., 2014; Scarpa et al., 1995) and is evolutionarily conserved across species (for a review, see: Gosling and John, 1999). Individuals with an inhibited temperament are shy, quiet, cautious, and withdraw from both social and non-social novelty. Inhibited temperament has been consistently shown to be a developmental risk factor for anxiety disorders (Biederman et al., 2001, 1993; Chronis-Tuscano et al., 2009; Clauss and Blackford, 2012; Essex et al., 2010; Hirshfeld et al., 1992; Schwartz et al., 1999).

Figure 1. Development of social anxiety disorder in children with an inhibited temperament.



Approximately 43% of children characterized as having an inhibited temperament (107/246) develop social anxiety disorder by mid-adolescence. In contrast, only about 13% of control children (57/446) develop social anxiety disorder by the same age. Data adapted from (Clauss and Blackford, 2012).

Psychiatric Findings

Children with an inhibited temperament are at increased risk for developing social anxiety disorder, overanxious disorder (also known as generalized anxiety disorder), specific phobias, and separation anxiety disorder (Biederman et al., 2001, 1993; Clauss and Blackford, 2012; Hirshfeld-Becker, 2010; Schwartz et al., 1999). Early studies showed that parents with panic disorder and agoraphobia were more likely to have inhibited children (Rosenbaum et al., 1988) and that inhibited children were more likely to have parents with a history of anxiety disorders (Rosenbaum et al., 1992, 1991). In a recent meta-analysis, 44% of children who were identified as inhibited as children developed social anxiety disorder by early adolescence, compared to just 13% of control children (see Figure 1; Clauss and Blackford, 2012). Inhibited temperament may have a specific relationship with social anxiety disorder based on the following evidence: 1) children who are continuously inhibited are more likely to develop social anxiety disorder (Chronis-Tuscano et al., 2009; Essex et al., 2010); 2) adolescents and adults who report themselves to be more inhibited as children are more likely to have social anxiety (Hayward et al., 1998; Neal et al., 2002); and 3) patients with social anxiety disorder report higher levels of childhood inhibited temperament than patients with other anxiety disorders (Gladstone et al., 2005; Mick and Telch, 1998). Inhibited temperament has been consistently associated with risk for anxiety disorders, specifically social anxiety disorder, and children who remain inhibited over time may be at highest risk.

Stability of Temperament

Children can become more or less inhibited over time, but children who remain continuously inhibited become more socially fearful and shy, and develop more internalizing problems, suggesting that stability of temperament may be critical to the development of social anxiety disorder (Fox et al., 2001). Inhibited temperament is moderately stable over both the short-term (3-5 weeks; Garcia-Coll et al., 1984) and long-term (years; Kagan and Moss, 1962; Kagan et al., 1998). Across several studies, infants who displayed motor and vocal reactivity to novelty and distress upon separation from their mothers (high reactivity) were more likely to be inhibited as toddlers and children (Calkins et al., 1996; Fox et al., 2001; Kagan et al., 1998). However, not all high-reactive children went on to develop inhibited temperament (Fox et al., 2001), and what influences children to become more or less inhibited over time remains unknown. The developmental trajectories of inhibited children are likely shaped by both nature, including underlying biological differences, and nurture, including differences in parenting, non-paternal care settings, and school environments. Further study of the developmental trajectories of inhibited children remains critical to determining which children will go on to develop psychiatric disorders and how these disorders can be prevented (Fox et al., 2013).

Measuring Temperament

Given that inhibited temperament is a strong risk factor for social anxiety disorder, the measurement of inhibited temperament may be useful for both

clinical settings and research studies. Inhibited temperament can be measured in a variety of ways. Early studies defined inhibited temperament by observing behavioral responses to novel stimuli in the laboratory, such as when a child cried or clung to his mother when encountering an unfamiliar experimenter (Garcia-Coll et al., 1984) or by counting the child's spontaneous comments to a novel experimenter to novel peers (Kagan et al., 1998). Behavioral measurements of temperament in the laboratory are standardized across children in the study and are not subject to reporter bias; however, they have limited utility as a screening measure, as they are expensive, time consuming, and may not reflect the child's behavior in daily life. Parent-report screenings are quick, can be completed in a variety of forms, and measure the child's behavior across different situations; however, these measures may be subject to reporter bias. In one study, maternal report of temperament, but not laboratory behavioral observations of temperament, was related to development of social anxiety disorder (Chronis-Tuscano et al., 2009), suggesting that parent-report may be a better predictor of psychopathology. While both behavioral observation and parent-report have their own strengths, identifying a brief, inexpensive screening method for inhibited temperament is critical to using inhibited temperament as a screening method for both clinical settings and research studies.

Biology of Inhibited Temperament

Sympathetic Nervous System

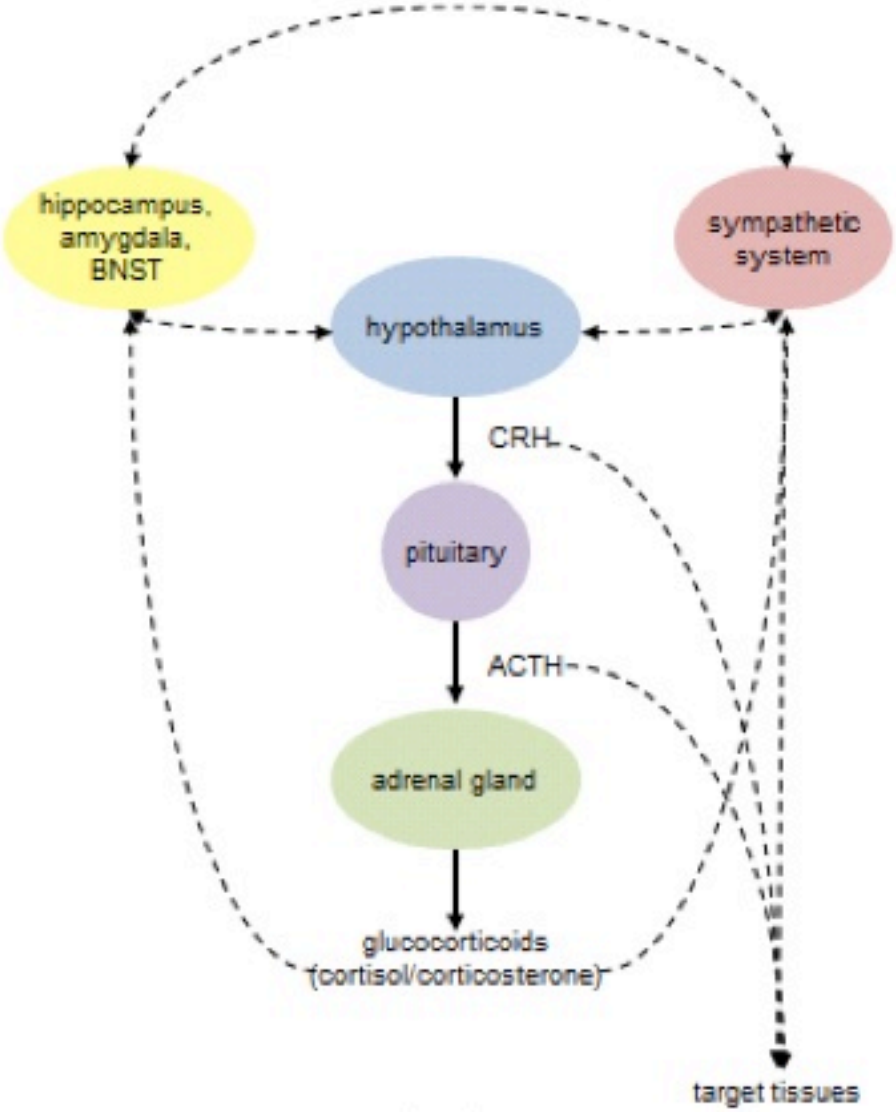
Inhibited temperament is associated with a lower threshold for activation of the sympathetic nervous system (Morris et al., 2005). Inhibited children have a higher and more stable (less variable) heart rate (Garcia-Coll et al., 1984; Kagan et al., 1988, 1987; Marshall and Stevenson-Hinde, 1998), implying less parasympathetic input, and inhibited children also have larger increase in heart rate in response to a drop in blood pressure (Kagan et al., 1988), implying a larger sympathetic response. Inhibited children also have increased sympathetic activity on other measures, including larger pupillary diameter, increased laryngeal muscle tension, and increased urinary norepinephrine concentration (Kagan et al., 1988, 1987). Inhibited children have a larger increase in anxious behavior and heart rate in response to giving a presentation in front of older peers (Schmidt et al., 1999). The sympathetic nervous system can be activated by the amygdala or other brain regions involved in response to fear or threat (see Figure 2).

Hypothalamic-Pituitary Adrenal Axis

The cortisol response is altered in many psychiatric disorders and is also implicated in inhibited temperament. Cortisol release coordinates the peripheral aspects of the stress response and can activate the sympathetic nervous system (see Figure 2; Lupien et al., 2009). Cortisol and other related hormones, such as

corticotropin-releasing hormone (CRH), feedback onto the brain to shut down the stress response. Most studies of the hypothalamic pituitary-adrenal (HPA) axis response in inhibited temperament have found that inhibited temperament is associated with higher cortisol at baseline and in response to stress. Inhibited children had significantly higher cortisol concentrations at home and in the laboratory (Kagan et al., 1987; Schmidt et al., 1997); however, see (Schmidt et al., 1999). Pérez-Edgar and colleagues (2008) found that inhibited temperament was associated with higher morning salivary cortisol and that cortisol concentration and negative affect predicted social withdrawal behavior, a precursor of social anxiety. Further investigation showed that cortisol concentration at 4 years was significantly associated with withdrawal behavior in boys with a history of high negative affect. High cortisol might sustain negative affect and inhibited behaviors in boys with high negative affect early in life. Children with an inhibited temperament may be more sensitive to social stressors, resulting in increased cortisol reactivity, this increased sensitivity to social stressors may be governed by alterations in amygdala, hippocampus, and bed nucleus of the stria terminalis (BNST) response.

Figure 2. Interaction of hypothalamic-adrenal nervous system with limbic system and sympathetic nervous system



The hypothalamic-pituitary-adrenal (HPA) axis interacts with the sympathetic nervous system and both systems are in a feedback loop with the amygdala and hippocampus, two regions implicated in inhibited temperament. Alterations of HPA axis function, sympathetic system function, and hippocampus and amygdala function have all been demonstrated in inhibited temperament and may be part of a critical loop underlying the temperament. For a review on HPA axis function, stress, and the brain, see (Lupien et al., 2009).

Neuroimaging of Inhibited Temperament

Functional Magnetic Resonance Imaging

Inhibited temperament was initially described as the tendency to avoid and be wary of novel people, places, or things. As the amygdala responds to novel, fearful, and salient stimuli in the environment (Blackford et al., 2010; Whalen, 1998), amygdala hyperactivity was first proposed as the neurobiological basis of inhibited temperament (Kagan and Snidman, 2004; Kagan et al., 1987). The first neuroimaging study of inhibited temperament (Schwartz et al., 2003a) confirmed a role for the amygdala in inhibited temperament and subsequent studies have clarified the nature of amygdala alterations. As research on the neurobiology of inhibited temperament has progressed, we have learned that inhibited temperament is also associated with critical differences in prefrontal cortex (PFC) activity and in amygdala-prefrontal neural circuits.

The majority of neuroimaging studies in inhibited temperament have used functional magnetic resonance imaging (fMRI). Functional neuroimaging allows us to gain insight into the human brain and to investigate how temperament may affect the brain's activity. FMRI also provides an opportunity to interrogate the timing and magnitude of the brain's response. Finally, fMRI can be used to understand the complex connectivity of the brain and test for differences in connectivity in inhibited temperament. FMRI has some disadvantages; it is highly dependent on the task design and so simple changes in order or timing may alter brain response. To date, 17 fMRI studies have been published in inhibited

temperament and these studies have expanded our understanding of inhibited temperament, from a trait characterized by heightened response to novelty, to a trait characterized by a sustained response to salient stimuli and a failure of emotion regulation to strong emotional stimuli.

Amygdala Findings

The initial characterization of inhibited temperament in children was based on increased reactivity to novelty (Garcia-Coll et al., 1984), and Kagan and others postulated that this increased reactivity was due to a hyperactive amygdala (Kagan and Snidman, 2004; Kagan et al., 1987). This hypothesis is consistent with evidence from animal and human studies showing that the amygdala rapidly detects novel, fearful, or salient stimuli and has efferent projections to other limbic areas, sensory regions, descending motor cortex, and PFC to prepare a response to the stimuli (Blackford et al., 2010; Ghashghaei et al., 2007; LeDoux et al., 1988; Whalen, 1998).

Based on the behavioral studies that established the importance of novel stimuli for eliciting inhibited behaviors, the earliest fMRI study examined brain responses to novel faces. In that study, Schwartz and colleagues (2003a) examined brain function in 22 young adults who had participated in a longitudinal study of inhibited temperament as toddlers (Garcia-Coll et al., 1984) and found that young adults with a history of inhibited temperament had greater amygdala activation to novel, compared to familiar faces. In contrast, the young adults with a history of uninhibited temperament had similar responses to both face types

(Schwartz et al., 2003a). Next, Blackford and colleagues (2009) tested for temperament differences in the temporal dynamics, or timing, of amygdala responses to novel and familiar faces. Compared to the uninhibited group, inhibited young adults had a faster amygdala response to novel faces and a longer amygdala response (duration) to both novel and familiar faces. These results show that inhibited subjects are more reactive to novelty and that less salient or ambiguous stimuli may engage the amygdala in inhibited subjects.

In a subsequent study, Blackford and colleagues (2011) examined the magnitude of amygdala responses to novel and familiar faces in inhibited and uninhibited young adults. The uninhibited temperament group had an increased amygdala activation to novel faces, but not to familiar faces; this pattern is consistent with findings that the human amygdala responds to novelty and then rapidly habituates (Blackford et al., 2010; Breiter et al., 1996; Fried et al., 1997; Schwartz et al., 2003b). In contrast, the inhibited group had increased amygdala activation to both the novel and familiar faces, suggesting that the inhibited temperament group continued to have an amygdala response to the familiar faces (Blackford et al., 2011).

Amygdala activation is altered in inhibited temperament and is modulated by attention state. Behaviorally inhibited adolescents had greater amygdala activation while rating subjective fear to emotional faces (Pérez-Edgar et al., 2007). This finding was consistent across all emotion types, including to happy faces. Fear rating induces internal reflection about feelings of fear and anxiety,

and in inhibited individuals, this internal reflection may provoke increased amygdala activation.

Reactivity and Regulation

While the initial definition of inhibited temperament was based on heightened reactivity to novelty, emerging evidence suggests that inhibited temperament is also based in a failure of reactivity to highly emotional stimuli. Mary Rothbart and others have postulated that temperament is based on differences in both reactivity and regulation (Rothbart, 1989). While an individual may be inhibited because he is highly reactive to novelty and other salient stimuli, he may also be inhibited because he lacks regulation of the reactivity. Reactivity is governed by the amygdala, and regulation is governed by the PFC, including the anterior cingulate cortex and dorsolateral PFC (Pérez-Edgar, 2014). The PFC has direct and indirect connections with the amygdala (Carmichael and Price, 1995; Ghashghaei et al., 2007; Ray and Zald, 2012) and can function to inhibit amygdala responses (Quirk et al., 2003; Rosenkranz and Grace, 2002). To test whether inhibited temperament is also associated with decreased regulation, it is necessary to use tasks that specifically engage the PFC, such as preparation for an aversive event, emotion regulation, or attentional control.

Prefrontal Cortex Findings

One way to interrogate regulatory processes is through cognitive control. Two recent studies examined the neurocircuitry of cognitive control in inhibited

temperament. First, Jarcho and colleagues (2013a) examined conflict monitoring and conflict adaptation. Conflict was created by presenting emotional faces labeled with the emotion on the face (congruent) or labeled with a different emotion (incongruent). During incongruent trials, which engage cognitive control mechanisms, compared with congruent trials, inhibited subjects had greater activation in the dorsomedial prefrontal cortex (dmPFC), insula, and parietal cortex. Finally, individuals with a history of psychopathology (across both temperament groups) had less dorsolateral prefrontal cortex (dlPFC) activation to incongruent trials, relative to congruent trials and did not exhibit conflict adaptation (faster response to an incongruent trial following an incongruent trial, relative to an incongruent trial following a congruent trial). In a second study, Jarcho and colleagues (2013b) tested for differences in attentional control, another form of cognitive control or regulation. Subjects saw male and female emotional faces that were labeled with the words “male” and “female”. High attentional control was measured as activity during gender-incongruent word-picture pairs and low attention control as measured as activity during gender-congruent word-gender pairs. During engagement of high attentional control while viewing fear faces, inhibited subjects had greater activation of the dmPFC, anterior cingulate cortex (ACC), dlPFC, precuneus, and basal ganglia. Using two different tasks to measure cognitive control mechanisms, inhibited adults had increased activity of a number of prefrontal regions, particularly the dmPFC, ACC, and dlPFC; however, subjects with anxiety disorders had less activity of the PFC.

Another way to measure emotion regulation is through anticipatory or expectancy processing. During anticipation of an aversive event, an individual can engage in adaptive behavior, such as planning and preparation, or the individual can engage in maladaptive behavior, such as worry and anxiety. Anticipation can differentially affect how individuals experience a situation or a stimulus. In the first study of anticipatory processing in inhibited temperament (Clauss et al., 2011), we manipulated the expectation of viewing fear faces. All participants viewed fear faces; however, half of the participants within each temperament group were warned that the fear faces would be shown and thus had an opportunity to prepare for viewing the faces (Expected group), the other half were not warned (Not-Expected group). Consistent with findings in healthy adults, the uninhibited participants in the Expected group showed stronger activation in the PFC regulatory regions (dorsal ACC and dlPFC) and decreased amygdala reactivity, compared to the uninhibited participants in the Not-Expected group. In the inhibited participants, the findings were opposite: those in the Expected group had reduced activation in prefrontal regulatory regions and increased amygdala reactivity relative to the inhibited participants in the Not-Expected condition. Thus, the inhibited group failed to effectively prepare for viewing the fear faces, and even showed evidence for a sensitization effect, where expectation produced an even stronger amygdala response.

The findings from the previous study suggested that individuals with an inhibited temperament engaged in different, and possibly maladaptive, anticipatory processes. Anticipation of upcoming aversive events is critical in

anxiety disorders; anxious individuals may have heightened arousal and may fail to effectively prepare for upcoming events, resulting in avoidance behavior (Grupe and Nitschke, 2013). However, the prior study was designed to test for differences in expectancy, and the anticipation period could not be separated from the face viewing period. In a subsequent study, (Clauss et al., 2014a) we examined anticipatory processing using a cued anticipation task. In the task, one cue signaled an upcoming fear face and another cue signaled an upcoming neutral face. Importantly, the time between the cue and face was sufficiently long to provide a reliable measure of anticipation. Temperament differences in fear anticipation were tested in a new sample of inhibited and uninhibited young adults. Contrary to the prior study, the inhibited adults had increased PFC activation during fear anticipation and no differences in amygdala activation. However, further examination within the inhibited group revealed that greater activation of the PFC was correlated with fewer social anxiety symptoms and better coping skills; these findings suggest that inhibited individuals who can effectively engage prefrontal regulation during anticipation of fear faces may be more resilient. This study also highlighted the utility of using an anticipatory processing paradigm to investigate temperament differences in both reactivity and regulatory processes.

Adults with an inhibited temperament have alterations in PFC-mediated regulation, and PFC activity may differentiate between inhibited adults who have developed social anxiety and those who have not. Inhibited subjects with more

PFC activation across two paradigms (anticipation and word-face conflict) had fewer anxiety symptoms.

Functional Connectivity Findings

Neuroimaging studies of inhibited temperament have clearly demonstrated that patterns of brain function in specific regions differ in adolescents and young adults who currently have an inhibited temperament or have a history of inhibited temperament. However, activity in one brain region influences activity in other regions. In order to fully understand the neurobiology of inhibited temperament and risk for social anxiety disorder, we must investigate neural circuits. The strength of connections between brain regions can be assessed using functional connectivity, a measure of the correlation or coherence of two brain regions. Functional connectivity can be measured during a task—task-based connectivity—or during a state of rest—resting-state or intrinsic connectivity.

To date, three studies have examined differences in functional connectivity in inhibited temperament: two tested for differences in task-based connectivity and one examined intrinsic connectivity. Hardee and colleagues (2013) tested for differences in amygdala connectivity during a dot-probe task in young adults with a history of inhibited temperament. The dot-probe task measures attention bias to threat stimuli. The inhibited group was significantly different from the uninhibited group in amygdala connectivity during angry trials, relative to neutral trials. During angry face trials, inhibited subjects had negative connectivity between the amygdala and dlPFC and between the amygdala and

insula; during neutral trials, inhibited subjects had positive connectivity between those regions. In comparison, the uninhibited group had similar connectivity across both conditions. Importantly, amygdala-insula connectivity mediated the relationship between childhood inhibition and internalizing symptoms in adulthood in inhibited subjects.

Clauss and colleagues (2014a) tested for differences in functional connectivity during anticipation of viewing fear faces, relative to neutral faces. Inhibited subjects had more negative connectivity between the amygdala and rostral ACC (rACC), between the insula and rACC and dorsal ACC (dACC), and between the insula and dlPFC during anticipation of viewing fear faces, relative to neutral faces. More positive connectivity between the amygdala and rACC was correlated with fewer social anxiety symptoms. Both the insula and rACC are connected to the amygdala and can both input and influence its output. Increased rACC output and functional connectivity with emotional brain regions, such as the insula and amygdala, may be protective against developing social anxiety symptoms in inhibited adults.

Finally, Blackford and colleagues (2014) tested for differences in intrinsic, or resting state, connectivity in inhibited young adults. The amygdala is a heterogeneous structure and amygdala subnuclei have distinct functions. Across all three amygdala subnuclei, a pattern emerged; inhibited individuals had reduced amygdala connectivity with regions that regulate the amygdala, such as the PFC, and with regions that are reciprocally connected with the amygdala, such as the hippocampus, visual cortex, and insula. Connectivity in several

resting state networks was also examined. Consistent with the amygdala findings, inhibited individuals had reduced connectivity between regions of the default mode network and dorsal attention network. In contrast, inhibited individuals had increased connectivity between regions in the executive control network and salience networks. Reduced amygdala connectivity may contribute to the amygdala habituation failure observed in prior studies (Blackford et al., 2013; Schwartz et al., 2012) and may underlie heightened avoidance behaviors. Increased connectivity in the executive control and salience networks may reflect heightened inhibitory control, the ability to activate or inhibit and override emotional responses.

Inhibited temperament is associated with alterations in amygdala-prefrontal connectivity and amygdala-insula connectivity. One critical challenge in the interpretation of connectivity studies is to understand if negative connectivity truly represents an inhibitory functional connection or if it is instead representative of “reduced” connectivity. Further research using animal models is needed to answer this question. We can, however, conclude that prefrontal modulation of amygdala activity may be critical to the development of anxiety symptoms in inhibited individuals.

Future Directions

Bed Nucleus of the Stria Terminalis

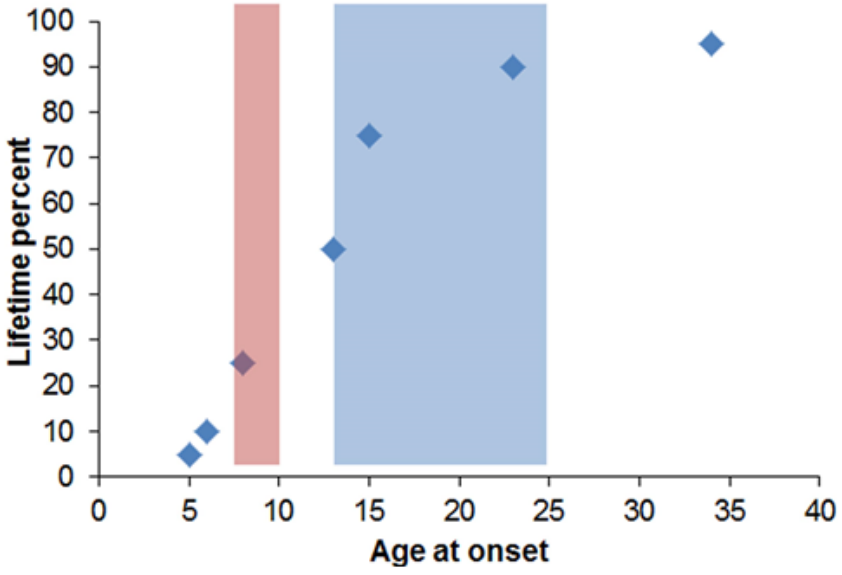
Emerging evidence from studies in humans and evidence from animal models has led us to propose that the bed nucleus of the stria terminalis (BNST) is critical to inhibited temperament. Based on work in both rodents and humans, the BNST plays a critical role in anxiety behavior and response to potential threat (Alvarez et al., 2011; Davis et al., 1997; Somerville et al., 2010; Walker and Davis, 2008) as well as in substance abuse (Flavin and Winder, 2013; Silberman and Winder, 2013; Stamatakis et al., 2014). In non-human primates, anxious temperament, the equivalent of inhibited temperament, was associated with activity of the BNST during a social novelty task (Fox et al., 2008) and OFC lesions alter both anxious temperament behavior and BNST activity during a social novelty task (Fox et al., 2010). The BNST is part of the "extended amygdala" and has strong structural and functional connections with the central nucleus of the amygdala (Avery et al., 2014; deCampo and Fudge, 2013; Dong and Swanson, 2006, 2004; Dong et al., 2001; Oler et al., 2012) To date, no human neuroimaging studies of inhibited temperament have explicitly tested for differences in BNST activity. There are a number of methodological challenges in examining BNST differences with fMRI. The BNST is a small structure (~1/10th the size of the amygdala) located in the basomedial forebrain and is difficult to delineate using conventional imaging methods. Recent advances in neuroimaging have allowed for smaller voxel sizes and thus more accurate

imaging of the BNST (Alvarez et al., 2011; Avery et al., 2014; Somerville et al., 2012, 2010). Tasks that activate the BNST, such as those that evoke uncertainty and potential threat, should be used in inhibited temperament to test for differences in BNST activation.

Neurobiological Alterations in Inhibited Temperament across Development

Inhibited temperament was initially identified in infants and toddlers (Garcia-Coll et al., 1984; Kagan et al., 1998), at present, all neuroimaging studies of inhibited temperament have been conducted in adolescents and young adults who were identified as infants (Schwartz et al., 2012, 2010) or as young children (Bar-Haim et al., 2009; Guyer et al., 2006; Hill et al., 2010; Jarcho et al., 2013a;

Figure 3. Age of onset of social anxiety disorder compared with ages of neuroimaging studies of inhibited temperament



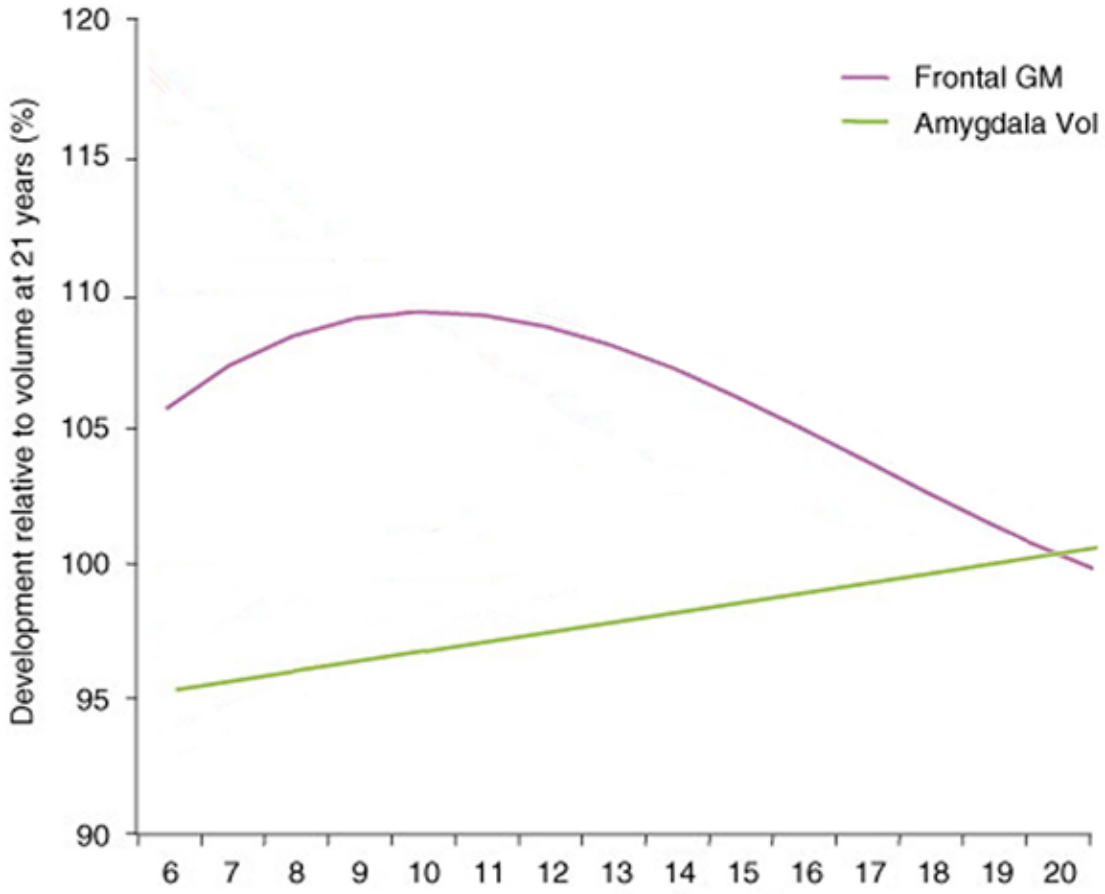
Social anxiety disorder has a median age of onset of 13 years and 75% of patients have an onset by age 15 (Kessler et al., 2005). Published neuroimaging studies of inhibited temperament have focused on imaging adolescents and young adults (blue), who have already reached the median age of onset for social anxiety disorder. Future studies should focus on younger children, who have yet to reach the age of onset for social anxiety disorder (red) to understand how temperament affects brain function.

Lahat et al., 2012; Pérez-Edgar et al., 2007) and followed longitudinally until imaging. Given that continuous inhibited temperament is associated with the highest risk for developing social anxiety disorder (Chronis-Tuscano et al., 2009; Essex et al., 2010), our lab has studied young adults who report being highly inhibited as children and young adults (Blackford et al., 2013, 2011, 2009; Clauss et al., 2014b, 2011; Edmiston and Blackford, 2013). Studying continuously inhibited young adults allows us to identify in young adults, the neurobiological alterations associated with inhibited temperament. Neurobiological alterations in adults with an inhibited temperament can then be tested for in young children with an inhibited temperament. Neuroimaging studies in inhibited temperament have focused on young adults and adolescents, with the earliest studies examining 13 year olds (see Figure 3). Social anxiety disorder has a median age of onset of 13 years, with 75% of patients developing social anxiety disorder by age 15 (Kessler et al., 2005). Additionally, inhibited temperament may contribute to result in an earlier age of onset of the disorder (Dalrymple et al., 2006; Rosellini et al., 2013). Future neuroimaging studies of inhibited temperament should examine younger children, prior to the age of onset of social anxiety disorder, to determine how temperament may affect the brain and how inhibited temperament may lead to social anxiety disorder.

Both the amygdala and PFC have been implicated in inhibited temperament and undergo developmental changes. The amygdala matures early in development and while amygdala volume increases over childhood (see Figure 4), increases in amygdala volume are due to increases in glial number,

the number of neurons stays relatively constant (Chareyron et al., 2012). In contrast, the PFC continues to mature throughout development, including periods of proliferation and pruning (Casey et al., 2000; see Figure 4; Gogtay et al., 2004; Huttenlocher, 2002).

Figure 4. Amygdala and prefrontal cortex development



The prefrontal cortex undergoes an early expansion and then gray matter volume decreases through adolescence. Amygdala volume undergoes a steady increase over time. Figure adapted from (Andersen and Teicher, 2008).

Few studies have examined developmental differences in amygdala-prefrontal networks and no studies have examined these changes in association with inhibited temperament. In the brain, broad connectivity changes over

development include strengthening of long-distance connections and weakening of short-distance connections (Supekar et al., 2009). Children ages 7-9 have weaker amygdala connectivity across a distributed network of both cortical and subcortical regions, notably, less functional connectivity with regulatory regions, including the rACC, dlPFC, and ventromedial PFC than young adults (Qin et al., 2012). Additionally, in the same study, functional networks across two amygdala subnuclei, the centromedial amygdala and the basolateral amygdala, were weaker and less segregated in children, implying a mechanism for immature emotional responses and less regulation of amygdala-based reactivity. In another sample, amygdala-medial PFC connections were positive in young children, and become more negative as children became adolescents, implying that as children enter adolescence, the medial PFC may inhibit amygdala reactivity (Gee et al., 2013b). This shift in connectivity is also accompanied by a developmentally-appropriate decrease in separation anxiety fears, and this connectivity shift may occur earlier in children who have experienced early stressors (Gee et al., 2013a, 2013b). Given that inhibited temperament has its basis in an alteration in both amygdala and PFC activity and connectivity, exploration of how this changes across development remains critical to understanding how inhibited temperament results in social anxiety disorder in some, but not all children. Functional segregation of these networks and increasing connectivity may be based on experiences and may provide an opportunity to intervene in at-risk children.

Summary

Inhibited temperament in adolescents and young adults is associated with alterations in amygdala reactivity and PFC regulation, and with less amygdala-PFC connectivity. Evidence from animal studies of anxiety and animal models of anxious temperament suggests that BNST reactivity may also be critical to the neurobiology of inhibited temperament. Emerging evidence suggests that increased PFC regulation may be protective or may promote resilience to social anxiety symptoms in inhibited children; however, that has yet to be tested. We propose that prior to the development of social anxiety disorder, inhibited children do not engage in preparatory anticipatory processing prior to viewing social stimuli, as measured by less PFC activation compared to uninhibited temperament children.

Specific Aims

The proposed aims are designed to identify neural correlates of risk for developing social anxiety disorder, including differences in brain activation during anticipation of viewing aversive social stimuli and differences in functional connectivity. The overall hypothesis is that children at high risk for developing social anxiety will have increased activation in emotional reactivity regions, such as the amygdala, insula, and BNST, and decreased activation in emotional modulatory regions, such as the dACC, dlPFC, and dmPFC. We also expect that children at high risk for developing social anxiety disorder will have decreased

connectivity between the amygdala and the dACC, both during an emotional task and during a non-emotional task.

To test these aims, we performed a cross-sectional study. We measured inhibited temperament and psychiatric symptoms by parent-report, self-report, and behavioral observation. We tested for differences in fMRI activation during a social anticipatory processing task and for differences in task-based functional connectivity and resting state connectivity. Using this design, we examine:

- differences in temperament and psychiatric symptoms in children with an inhibited or uninhibited temperament (Chapter 2)
- differences in brain activity during anticipation of social stimuli in children with an inhibited temperament (Chapter 3)
- alterations in amygdala-dACC connectivity in children with an inhibited temperament (Chapter 4)
- alterations in BNST connectivity in children with an inhibited temperament (Chapter 5)

CHAPTER II

SELECTION AND PHENOTYPING OF INHIBITED CHILDREN

Introduction

Inhibited temperament is the tendency to withdraw from or avoid new situations and new people; inhibited children represent the 15-20% of children who are most extreme on this measure (Garcia-Coll et al., 1984). Inhibited children are typically slow to warm up when they meet a new person, whether a new adult or a new peer (Garcia-Coll et al., 1984). They are more likely to play on the outskirts or observe a group of children, rather than join in their games (Kagan et al., 1984). Another component of inhibited temperament is risk aversion—inhibited children avoid taking physical risks, like jumping off from tall objects, and are more likely to follow their parents' rules (Kagan and Moss, 1962). Inhibited temperament consists of inhibition to both social and non-social situations and can be measured in a variety of ways.

Traditionally, inhibited temperament was measured in the laboratory by trained observers (for examples, see: Garcia-Coll et al., 1984; Kagan et al., 1984). Temperament assessments were specific to cognitive, social, and sensory abilities at each age. Experimenters presented novel social and non-social stimuli to the child, such as a colored mobile or an unfamiliar person in the room for an infant or a toddler, or a talking robot or cognitive testing for older children. For children in middle childhood, the temperament assessment often included an

interview with the child, with the interviewer asking a series of open-ended questions. The child's behavior was then rated by an objective rater. Duration and latency of behaviors can be quantified, such as duration of speaking or latency to approach an unfamiliar peer. Of all of the measures of temperament in the laboratory, latency to speak to an unfamiliar person is the most reliable (Kagan and Snidman, 2004). Laboratory-based measurements have many strengths; they are objective and can be standardized across children. However, laboratory measures also have limitations: they are expensive, time-consuming, state-dependent, and use an artificial environment (Morris et al., 2005).

Parent-report questionnaires are another common method of measuring temperament in children. Parent-report questionnaires ask parents to rate their child's frequency of inhibited behavior across several social and non-social contexts, such as "my child is shy when first meeting new children" or "my child is hesitant to explore new play equipment" (Bishop et al., 2003). These questionnaires are advantageous for use as a screening method, as they can be completed by mail or through an online form, are quick, and can be administered to a large number of parents at once. Parent-report questionnaires have many strengths; they report on the child's behavior across a variety of situations and in the child's natural environment. They also have limitations: they are subjectively based on the parent's experience, the parent's feelings about their child's temperament, and clinical state effects.

The Behavioral Inhibition Questionnaire (BIQ) is an excellent questionnaire for selecting inhibited children in middle childhood. The BIQ has

been validated in several non-clinical samples in children ages 3-15 (Bishop et al., 2003; Broeren and Muris, 2009; Edwards et al., 2010) and has demonstrated good reliability (12 month stability; maternal report: r = .78; paternal report: r = .74) and validity (maternal report and observer rating during behavioral assessment: r = .46; paternal report and behavioral assessment: r = .25; Bishop et al., 2003). Additionally, the BIQ has a validated child self-report form, with good parent-child agreement (r = .59; p < .001; Broeren and Muris, 2009). The BIQ also has predictive validity—it correlates with current anxiety symptoms (Broeren and Muris, 2009) and predict the development of anxiety symptoms 12 and 24 months later (Broeren et al., 2013; Edwards et al., 2010). Finally, BIQ scores were specifically related to symptoms of social anxiety, (Broeren and Muris, 2009) and initial BIQ scores differentiated between children who would have high social anxiety symptoms from children who would have few social anxiety symptoms over two years (Broeren et al., 2013).

We selected children based on being in the top 15% or bottom 15% of BIQ scores, based on normative data (Bishop et al., 2003; Broeren and Muris, 2009; van Brakel et al., 2004) who had no current or past psychopathology. To fully characterize the phenotypes of these children, we collected a number of other measures of temperament, as well as measures of anxiety, depression, and autism symptoms. Because we were interested in studying children with an inhibited temperament who had yet to develop anxiety disorders, we expected that our children rated as inhibited by their parents on the BIQ parent version (BIQ-P) would have higher anxiety symptoms than those rated as uninhibited, but

the symptoms would not have reached the diagnostic threshold of symptom reports clinical populations.

Methods

Recruitment and Screening

Children were recruited by advertisements looking for children with an extreme temperament “shy or outgoing”, “quiet or cautious”, or looking for children for a temperament and MRI study. Children were recruited from the Vanderbilt community using flyers, online recruitment databases, healthy controls from other research studies, and referrals from study participants.

Interested parents completed an online screening, which included a validated parent-report temperament questionnaire, the BIQ-P (Bishop et al., 2003), questions about MRI compatibility, and cognitive screening criteria. As with our past recruitment of young adults, we defined the two extreme temperament groups based on the top/bottom 15% of the population, as measured by being more extreme the average $\pm$ one standard deviation (inhibited > 123; uninhibited < 59). The online screening also included questions about inclusion/exclusion criteria (age, psychotropic medication use, psychiatric diagnosis, MRI exclusion, needing help in school, repeated grades).

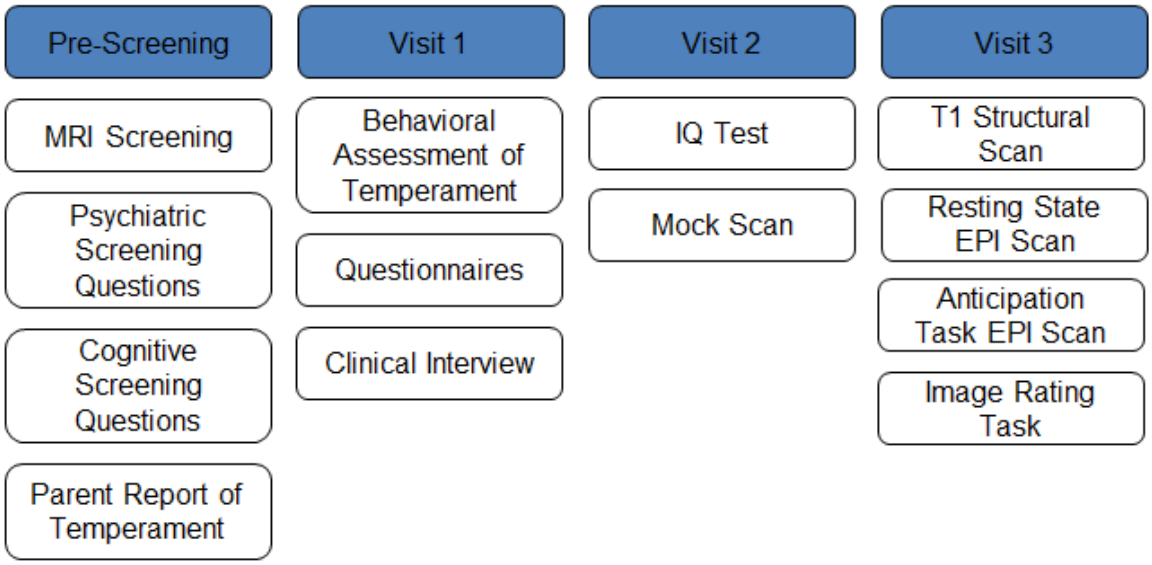
We selected children ages 8-10 years for the study for the following reasons: 1) being able to reliably complete an fMRI scan—based on a large-scale study, children between 8-10 have a 90% success rate in completing

functional MRI scans (Byars et al., 2002); 2) age of onset of social anxiety disorder—50% of patients have onset of their social anxiety disorder by age 13, whereas only 25% have an onset before age 8 (Kessler et al., 2005); 3) pilot data from our lab showing that children ages 11-12 had an increased prevalence of anxiety disorders compared to children ages 8-10 (Clauss and Blackford, unpublished); and 4) children 10 and under are less likely to have undergone puberty and have yet to enter middle school, two major life changes that are also associated with social anxiety (Blumenthal et al., 2009; Erath et al., 2007).

A variety of exclusion criteria were used in the study. To obtain a pure "at-risk" group, children were excluded from the study having any current or past psychiatric diagnoses or receiving treatment for anxiety symptoms (see below). To ensure that all children could complete study tasks, children were excluded for developmental delay, repeated grades, or needing special help in school. Finally, children were excluded for any contraindications for MRI, including metal in the body, claustrophobia, or medical conditions which might affect blood oxygen-level-dependent (BOLD) signal, including any current psychotropic medication use (past six months), history of head injury, major medical illness, or neurological illness. These exclusion criteria were initially assessed in the online parent screening and were re-assessed at the first study visit.

The Vanderbilt University Institutional Review Board approved the study and informed consent was obtained from parents and informed assent was obtained from children prior to participation.

Figure 5. Study protocol by visit.



Study Protocol

The study included three visits: 1) consent, behavioral assessment of temperament, clinical interview, and questionnaires; 2) completion of any remaining questionnaires, intelligence quotient (IQ) testing, and mock MRI scan; and 3) MRI scan and image rating task (see Figure 5).

Behavioral Assessment of Temperament. Following informed assent and consent, children were brought into a new room and were told that a "new experimenter was going to come in soon and ask them some questions". An unknown female experimenter entered the room and asked the child a series of open-ended questions about familiar topics (i.e. "I'd like to hear about your

school, can you tell me about your school?" or "Now I'd like to hear about your friends. Can you tell me about your friends?"). After the child finished talking, the experimenter waited 10 seconds before asking a follow-up question. If the child did not respond to the question, the experimenter paused for 10 seconds before repeating the question once. The interview lasted between 10-15 minutes for each child. Following the interview, the experimenter rated global inhibited temperament and seven other measures (length of time to answer questions, amount of speech, tense or uncomfortable behaviors, positive affect, negative affect, trust, and volume and tone of voice) on a 1-5 Likert scale, based on Ballespí et al. (2013).

Clinical Interview. All children underwent a structured diagnostic interview with the Kiddie Schedule for Affective Disorders and Schizophrenia (KSADS). To provide a more comprehensive assessment of anxiety, questions from the Anxiety Disorders Interview Schedule for Children (ADISC; Silverman and Nelles, 1988) were also asked. Both the KSADS and ADISC have demonstrated reliability and validity (Kaufman et al., 1997; Silverman and Nelles, 1988) and the KSADS is considered the gold-standard for pediatric diagnostic psychiatric interviews. This clinical interview protocol was developed by Dr. Danny Pine from the National Institute of Mental Health. The interviews were conducted by J.A.C., who was trained by Dr. John Weir, a clinical psychologist, Dr. Margaret Bennningfield, a child and adolescent psychiatrist, and Dr. Danny Pine, a child and adolescent psychiatrist, to conduct pediatric clinical interviews. J.A.C. consulted with Dr. Margaret Benningfield and Dr. Jennifer Blackford on

questionable interviews. Parent interviews were conducted first and were always completed on the first study visit. Child interviews were conducted second and were usually completed on the first study visit.

Temperament and Psychiatric Symptom Questionnaires. To further characterize the children, parents and children completed several additional questionnaires (see Tables 1 and 2). Parents completed the Retrospective Assessment of Behavioral Inhibition in Infants and Toddlers (RIBI; Gensthaler et al., 2012) and the Temperament in Middle Childhood Questionnaire (TMCQ; Simonds et al., 2007). Children completed the Behavioral Inhibition Questionnaire – Child version (BIQ-C; Broeren and Muris, 2009) and the Child

Table 1. Parent-report questionnaires

Questionnaire	Measures	Reliability and validity
Behavioral Inhibition Questionnaire – Parent	inhibited temperament	internal consistency (α = .67-.95) 12 month stability (r = .74 - 78)
Retrospective Infant Behavioral inhibition Scale	infant temperament	internal consistency (α = .92) mother-father agreement (total score: r = .71)
Conners 3 Parent Report – Short	symptoms of ADHD and externalizing disorders	internal consistency (α = .77 - .79) two to four week reliability (r = .71 - .98)
Screen for Child Anxiety Related Emotional Disorders – Parent version	general anxiety symptoms	internal consistency (α = .74 - .89) five week stability for parent and child-report (total score: r = .86) parent-child correlation (total score: r = .33)
Social Communication Questionnaire	autism symptoms	internal consistency (total score: α = .90) correlates with autism diagnostic interview (r = .71) discriminates between autism and controls (t = 11.01)
Temperament in Middle Childhood Questionnaire	16 dimensions of temperament, including inhibitory control, fear, shyness	internal consistency (α = .69 - 90)

Self-Report of Inhibition (CSRI; adapted from Reznick et al., 1992). To provide dimensional measures of anxiety symptoms, children completed several self-report measures of anxiety, including the Screen for Child Anxiety-Related Emotional Disorders (SCARED; Birmaher et al., 1997), the Social Phobia and Anxiety Inventory for Children (SPAI-C; Beidel et al., 1995), and the Multidimensional Anxiety Scale for Children (MASC; March et al., 1997). Because children may have difficulty assessing the frequency and severity of their anxiety symptoms, parents also completed the parent version of the SCARED. Inhibited temperament is also associated with symptoms of autism and depression (Caspi et al., 1996; Gladstone and Parker, 2006); to measure autism symptoms, parents completed the Social Communication Questionnaire (SCQ; Berument et al., 1999; Rutter et al., 2003) and children completed the Children's Depression Inventory (CDI; Smucker et al., 1986). Finally, uninhibited temperament is associated with attention deficit hyperactivity disorder (ADHD) symptoms and externalizing disorders (Hirshfeld-Becker et al., 2007, 2003), so parents completed the Conners 3 Parent Rating Scale (Conners et al., 1998). Subscale scores on the Conners were converted to t-s cores using gender and age-specific criteria.

Intelligence Quotient Testing. To ensure that there were no significant differences in comprehension of the task or significant differences in reading ability, children completed the Kaufman Brief Intelligence Test, second edition (KBIT; Kaufman and Kaufman, 1990). The KBIT was chosen because it is brief (15-30 minutes to administer), is designed for individuals between 4-90 years old, and it has good external validity (Canivez, 1995), it correlates well with other similar IQ tests, such as the KBIT Composite and Wechsler Full-Scale IQ (r = .61-.88; Spreen and Strauss, 1998). The KBIT was administered by JAC during the second study visit.

Table 2. Child self-report questionnaires

Questionnaire	Measures	Reliability and validity
Behavioral Inhibition Questionnaire – Child version	inhibited temperament	parent-child agreement (total score: r = .59) internal consistency (α = .91)
Child Self-Report of Inhibition	inhibited temperament	internal consistency (α = .62-.73) test-retest reliability (r = .59-.68) correlates with fear and distress subscales from emotionality, activity, and sociability instrument
Screen for Childhood Anxiety-Related Emotional Disorders – Child	general anxiety symptoms	internal consistency (α = .74 - .89) five week stability for parent and child-report (total score: r = .86) parent-child correlation (total score: r = .33) significantly differentiated children with anxiety disorders from disruptive and depressive disorders (t = 6.54)
Multi-dimensional Anxiety Scale for Children	general anxiety symptoms	internal consistency (total score: α = .90) three month stability (total score: r = .93) significantly correlated with Revised Children's Manifest Anxiety Scale (r = .63)
Social Phobia and Anxiety Inventory for Children	social anxiety symptoms	internal consistency (α = .95) two week reliability (r = .86) differentiated children with social anxiety (t = 2.92)
Children's Depression Inventory	depression symptoms	internal consistency (α = .84 - .87) one year reliability (r = .41 - .69)

Data Analysis

Internal Consistency. To confirm that measures used in the study were internally consistent, and thus were a reliable measure of temperament or symptoms, Cronbach's alpha was calculated for each measure included in the study.

Temperament Validation. To validate that the BIQ-P was selecting children with an inhibited or uninhibited temperament, we also tested for differences on child self-report of temperament (CSRI and BIQ-C) and two other parent-report measures of temperament (RIBI and TMCQ). We also tested to see if children were different in behavioral assessment of temperament by an objective interviewer.

Psychiatric Symptom Validation. To determine if we were selecting inhibited children at risk for anxiety disorders, but who had yet to develop anxiety disorders, we tested for group differences in anxiety symptoms. Finally, we tested to see if inhibited children and uninhibited children differed on symptoms of depression, ADHD, and autism. While inhibited temperament is associated with autism and depression and uninhibited temperament is associated with ADHD, we did not expect to see differences on these measures. ADHD and autism typically have their onsets early in life (ADHD: median onset 7 years; autism: median onset 3 years; Kessler et al., 2005; Mandell et al., 2005), and we were specifically excluding children with those disorders. Depression typically has its onset later in life (median onset 31 years; Kessler et al., 2005) and we would not expect inhibited children to have yet to develop depression, especially

as it is often secondary to the development of social anxiety disorder (Beesdo et al., 2007).

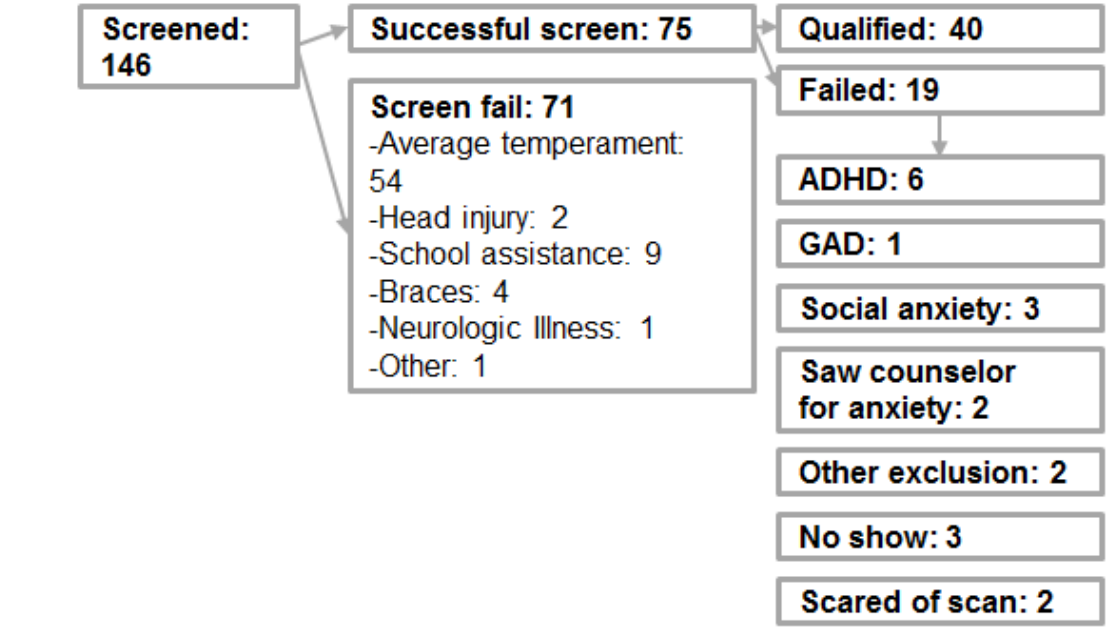
Statistical Analysis. All statistical tests were performed in SPSS (version 21.0.0.0). Correlations between parent and child-report were calculated using a two-tailed bivariate correlation. Group differences in continuous variables were tested using two-sample t-tests. Differences in categorical variables were calculated using a chi-square test. All tests were considered significant at $\alpha = .05$.

Results

Recruitment

Parents of 146 children completed the online screening (see Figure 6). Of those children, 75 children (51.3%) passed the initial screening and 71 children failed the initial screening. Children failed for a variety of reasons, most commonly not having an extreme temperament score (n = 54). The other children failed for a variety of exclusion criteria, including needing special assistance in school (n = 9), having braces (n = 4), history of head injury (n = 2), history of neurological illness (n = 1), and other parent concerns (n = 1). Of the 75 children who passed the initial screening, 59 (78.7%) were seen in the lab for a first study visit. Forty children who were seen for the initial lab visit qualified for the study and completed all three study visits. Nineteen children either did not qualify or did not complete the study for a variety of reasons: meeting criteria for a psychiatric

Figure 6. Flowchart of screening and subject exclusion



ADHD = attention deficit hyperactivity disorder; GAD = generalized anxiety disorder

disorder (ADHD: n = 6; social anxiety disorder: n = 2; generalized anxiety disorder: n = 1); having sought treatment for anxiety symptoms (n = 2); meeting initial exclusion criteria (i.e. having braces, being outside of age range; n = 2); being too scared to complete the MRI scan (n = 2); and missing more than one study appointment (n = 3).

Demographics

Forty subjects were enrolled in the study (20 inhibited, 20 uninhibited). Subjects were on average 9.7 years old when they were scanned (SD: 1.0 years, see Table 3). Subjects were 90% Caucasian, 5% Asian, and 5% African-American. Sixty percent of subjects were female and 90% were right-handed (using the Edinburgh Handedness Inventory). Average IQ was 116.1 (SD: 10.6).

There were no significant differences between the two temperament groups on age, ethnicity, handedness, or IQ.

Table 3. Demographic data

	IT n = 20		UT n = 20		p-value
	Mean	**SD**	**Mean**	**SD**	
Age (years)	9.5	1.1	9.8	.9	.39
IQ	116.2	9.2	116.0	12.0	.92
	N	**(%)**	**N**	**(%)**	
Ethnicity					.99
Caucasian	18	90%	18	90%	
African-American	1	5%	1	5%	
Asian	1	5%	1	5%	
Female Gender	12	60%	12	60%	.64
Right Handed	18	90%	18	90%	.99

Note: IT = inhibited temperament; UT = uninhibited temperament; SD = standard deviation; IQ = intelligence quotient

Internal Consistency

The BIQ, CSRI, RIBI, SCARED, MASC, SPAI, SCQ, and CDI all had adequate internal consistency, ranging from .66 to .99 (see Table 4; Cortina, 1993). The TMCQ had adequate internal consistency for all subscales except for the low-intensity pleasure and shyness subscales, which had alphas of .55 and .50, respectively. The Conners 3 Parent Report had good consistency, except for one scale, the defiance/aggression subscale scale, which had an alpha of .51.

Table 4. Internal consistency and parent-child correspondence

Questionnaire	Parent-report α	Child-report α	Parent-child correlation
BIQ	.99	.89	.41
CSRI	NA	.81	NA
RIBI	.93	NA	NA
TMCQ	Activation Control .75 Activity Level .91 Affiliation .76 Anger/Frustration .77 Assertiveness/Dominance .82 Attention Focusing .84 Discomfort .79 Fantasy/Openness .74 Fear .85 High Intensity Pleasure .85 Impulsivity .85 Inhibitory Control .75 Low Intensity Pleasure .55 Perceptual Sensitivity .67 Sadness .85 Shyness .50 Soothability/Falling Reactivity .79	NA	NA
SCARED	.94	.95	.23
MASC	NA	.91	NA
SPAI-C	NA	.96	NA
CDI	NA	.85	NA
Conners 3	Inattention .75 Hyperactivity/Impulsivity .80 Executive Functioning .66 Peer Relations .68 Defiance/Aggression .51	NA	NA
SCQ	.68	NA	NA

Note: BIQ = Behavioral Inhibition Questionnaire; CSRI = Child Self-Report of Inhibition; RIBI = Retrospective Infant Behavioral Inhibition Scale; TMCQ = Temperament in Middle Childhood Questionnaire; SCARED = Screen for Child Anxiety-Related Emotional Disorders; MASC = Multidimensional Anxiety Scale for Children; SPAI = Social Phobia and Anxiety Inventory for Children; CDI = Children's Depression Inventory; SCQ = Social Communication Questionnaire; NA = not applicable

Parent-Child Correspondence

Two scales had a parent and a child version, the BIQ and the SCARED. Parents and children were moderately correlated on BIQ score (r = .41, p = .003;

see Table 4); these findings were confirmed using non-parametric testing (Spearman's rho = .44, p = .004). On the subscales of the BIQ, parents and children correlated on subscales of behavioral inhibition to peers (r = .35, p = .012), physical (r = .28, p = .046), separation fears (r = .31, p = .029), performance fears (r = .41, p = .003), behavioral inhibition to adults (r = .40, p = .004), and unfamiliar (r = .41, p = .003). On total SCARED total score, there was only a trend in correlation between parent and child-report (r = .23, p = .12). The two groups were correlated on SCARED subscales of separation anxiety (r = .34, p = .02) and social anxiety (r = .47, p = .001), but not on panic disorder, generalized anxiety disorder, or school fears (all p > .22).

Temperament Measures

Parents of inhibited children rated their children as more inhibited during infancy and toddlerhood using the RIBI (mean $\pm$ SD; IT: 38.4 $\pm$ 13.2; UT: 66.5 $\pm$ 5.6; p < .001; see Table 5) and as having a more fearful (IT: 2.9 $\pm$ 0.7; UT: 1.8 $\pm$ 0.5; p < .001) and shy temperament (IT: 3.7 $\pm$ 0.7; UT: 1.6 $\pm$ 0.4) than the uninhibited children using the TMCQ. Inhibited children rated themselves as more inhibited than the uninhibited children using the BIQ-C (IT: 122.9 $\pm$ 19.2; UT: 95.1 $\pm$ 37.5; p = .005), but not using the CSRI (IT: 2.3 $\pm$ 0.4; UT: 2.0 $\pm$ 0.6; p = .15). Interviewers rated the inhibited children as more inhibited than the uninhibited children (IT: 3.4 $\pm$ 1.1; UT: 2.2 $\pm$ 1.2; p = .002).

Table 5. Temperament measures

	IT		UT		p-value
	Mean	SD	Mean	SD	
BIQ-P	153.8	20.2	44.7	8.5	< .001
BIQ-C	122.9	19.2	95.1	37.5	.005
RIBI	38.4	13.2	66.5	5.6	< .001
TMCQ-Fear	2.9	0.7	1.8	0.5	< .001
TMCQ-Shy	3.7	0.7	1.6	0.4	< .001
CSRI	2.3	0.4	2.0	0.6	.208
Interviewer rating	3.4	1.1	2.2	1.2	.002

Note: IT = inhibited temperament; UT = uninhibited temperament; SD = standard deviation; BIQ-P = Behavioral Inhibition Questionnaire – Parent report; BIQ-C = Behavioral Inhibition Questionnaire – Child report; RIBI = Retrospective Infant Behavioral Inhibition Scale; TMCQ = Temperament in Middle Childhood Questionnaire; CSRI = Child Self-Report of Inhibition

Psychiatric Symptoms

As expected, inhibited children were rated by their parents as more anxious using the SCARED-P (mean ± SD; IT: 21.8 ± 8.4; UT: 4.2 ± 3.5; $p < .001$; see Table 6) and reported themselves to have more global anxiety, as measured by the SCARED-C (IT: 19.5 ± 11.8; UT: 11.1 ± 10.8; $p = .03$) and to have more social anxiety, as measured by the SPAI-C (IT: 19.7 ± 11.2; UT: 12.4 ± 7.9; $p = .02$), but not the MASC (IT: 55.9 ± 14.7; UT: 51.1 ± 18.5; $p = .37$). Reports of symptoms other than anxiety did not differ between the groups; for example the two groups had a trend towards significant differences in symptoms of inattention (IT: 47.1 ± 6.1; UT: 50.4 ± 5.1; $p = .07$) but did not significantly

differ on parent report of other ADHD symptoms, including: hyperactivity (IT: 49.9 ± 8.8; UT: 51.7 ± 10.8; p = .57); executive functioning (IT: 49.5 ± 8.8; UT: 52.5 ± 9.1; p = .24); defiance/aggression (IT: 46.7 ± 3.8; UT: 48.0 ± 8.1; p = .92). The two groups did not differ on parent- report of symptoms of autism (IT: 4.0 ± 3.7; UT: 2.7 ± 2.9; p = .17) or child self-report of symptoms of depression (IT: 5.7 ± 3.2; UT: 5.4 ± 7.4; p = .88).

Table 6. Psychiatric symptom measures

	IT		UT		p-value
	Mean	SD	Mean	SD	
Anxiety Symptoms					
SCARED-P	21.8	8.4	4.2	3.5	< .001
SCARED-C	19.5	11.8	11.1	10.8	.03
MASC	55.9	14.7	51.1	18.5	.51
SPAI-C	19.7	11.2	12.4	7.9	.02
Other Symptoms					
Conners – Inattention (t-value)	47.1	6.1	50.4	5.1	.05
Conners – Hyperactivity (t-value)	49.9	8.8	51.7	10.8	.39
Conners – Executive Functioning (t-value)	49.5	6.7	52.5	9.1	30
Conners – Defiance/Aggression (t-value)	46.7	3.8	48.0	8.1	.56
Conners – Peer Relations (t-value)	56.2	14.9	51.5	8.1	.10
SCQ – Autism	4.0	3.7	2.7	1.9	.11
CDI – Depression	5.7	3.2	5.4	7.4	.90

Note: SCARED-P = Screen for Child Anxiety Related Emotional Disorders Parent Version; SCARED-C = Screen for Child Anxiety Related Emotional Disorders Child Version; MASC = Multidimensional Anxiety Scale for Children; SPAI-C = Social Phobia and Anxiety Inventory for Children; SCQ = Social Communication Questionnaire; CDI = Children's Depression Inventory; ns = not significant; SD = standard deviation

Discussion

The aim of this study was to successfully recruit inhibited children who were at high risk for developing anxiety disorders and uninhibited children who were at low risk for developing anxiety disorders using a parent questionnaire measure, the Behavioral Inhibition Questionnaire (BIQ-P). Forty children (20 inhibited, 20 uninhibited) were enrolled in the study. The two groups of children were significantly different on other parent-report, child self-report, and behavioral observations of temperament. Inhibited children had higher levels of non-clinical anxiety, but did not differ in symptoms of autism, ADHD, or depression from their uninhibited peers. This study demonstrates that inhibited children can be easily selected using a brief online parent-report screening questionnaire. Validation that the BIQ-P selects at-risk children is critical for future studies of anxiety risk.

Temperament may be best measured using multiple raters and situations (Kraemer et al., 2003; Pérez-Edgar and Guyer, 2014). Parent report and interviewer reports of inhibited temperament were moderately correlated; however, child self-report did not correlate with interviewer rating (see Figure 8). These findings suggest that both behavioral and parent observations may measure external behaviors and may be better at measuring children in relation to others, whereas children may lack insight into their own level of shyness. As children enter adolescence, they become more aware of their behavior in relation to their peers (Morris et al., 2005). Parents and children were moderately correlated on the total BIQ score; the parent and child versions of the BIQ

contained the same questions (modified for the appropriate reporter), and so it would be expected that parents and children would respond similarly to the questions.

Our interest in inhibited temperament is primarily as a risk factor for the development of social anxiety disorder. Inhibited children selected using the BIQ-P had significantly higher anxiety symptoms than the uninhibited group, but importantly, the inhibited children did not meet criteria for a psychiatric diagnosis. In addition, the inhibited group's mean score was lower than SCARED scores in treatment-seeking children with anxiety disorders (parent: 36.6 $\pm$ 13.4; child total score mean: 31.6 $\pm$ 13.4; Walkup et al., 2001). Similarly, the social anxiety scores for the inhibited children were higher than the uninhibited children, but still lower than children with diagnosed social anxiety disorder (26.1; Beidel et al., 2001). Given that this group has not yet reached the median age of onset of social anxiety disorder, but have increased symptoms, they are likely to be at increased risk for developing social anxiety disorder.

Inhibited temperament is a risk factor for anxiety disorders, most notably social anxiety disorder, but is also associated with symptoms of autism and depression and uninhibited temperament is associated with symptoms of ADHD (Hirshfeld-Becker et al., 2007, 2003). Parents of children in both temperament groups reported few autism symptoms and group means were well below the recommended screening cut-off of 15 (Chandler et al., 2007). Children in both temperament groups also reported few depressive symptoms, and group mean

were below the screening cut-off of 16 (Timbremont et al., 2004). Finally, parents of both inhibited and uninhibited children reported few ADHD symptoms.

This study had several limitations. First, children were recruited for extreme temperament, and did not include children with an average temperament, preventing us from drawing conclusions about the utility of the BIQ-P as a screening measure for average temperament children. However, prior studies have found significant correlations across the entire range of temperament between parent-report using the BIQ-P and behavioral observations of temperament (Bishop et al., 2003). Second, inhibited children did not differ on their self-report using the CSRI; however, there was a trend-level difference between the two groups. The CSRI did correlate with BIQ-C scores ($r = .70$; $p < .001$) and had a trend towards correlation with parent-report scores ($r = .28$; $p = .08$), but did not correlate with interviewer scoring ($r = -.03$, $p = .84$). The CSRI correlated with several subscales of the BIQ-C, including inhibition to peers ($r = .66$, $p < .001$), physical fears ($r = .49$, $p = .001$), separation fears ($r = .65$, $p < .001$), inhibition to adults ($r = .56$, $p < .001$), and inhibition to unfamiliar people ($r = .53$, $p < .001$). The CSRI correlated with only one subscale of the BIQ-P, separation fears ($r = 42$, $p = .008$). These findings suggest that the CSRI may measure the child's internal feelings, rather than external behaviors. Third, some questionnaires had poor internal consistency, including several subscales of the TMCQ and the defiance subscale of the Conners; however, it should be noted that the size of Cronbach's α varies with the length of the measure, with longer

measures having higher Cronbach's α (Cortina, 1993). All three scales that had poor internal reliability also had few questions (5-8 questions/subscale).

Conclusions

In conclusion, we successfully used the BIQ-P to select inhibited children who are at risk for developing anxiety disorders. Traditionally, inhibited temperament has been measured using a behavioral measure of temperament, and that has sometimes been combined with parent or child-report questionnaires in older children. Behavioral measures are expensive, time-consuming, and are not feasible for large scale screening. In contrast, using the BIQ-P, we identified inhibited and uninhibited children who were also rated by behavioral observers as being significantly different from each other. Parent-ratings using the BIQ-P and interviewer ratings were significantly correlated, suggesting that the BIQ-P is measuring some of the same constructs as the behavioral interview. Future studies should examine whether it is useful as a clinical screening tool in primary care settings and for large-scale longitudinal research studies.

CHAPTER III

NEURAL CORRELATES OF ANTICIPATION OF SOCIAL STIMULI IN CHILDREN AT HIGH RISK FOR SOCIAL ANXIETY DISORDER

Introduction

Inhibited temperament has been consistently associated with increased amygdala activation and with alterations in prefrontal cortex activation (Blackford et al., 2013, 2011; Clauss et al., 2014b, 2011; Jarcho et al., 2013a, 2013b; Pérez-Edgar et al., 2007; Schwartz et al., 2012, 2003a).The prefrontal cortex inhibits amygdala response (Quirk et al., 2003) and prefrontal cortex activity may be important for the development of social anxiety disorder in inhibited temperament, as inhibited adults with less prefrontal cortex activity have more anxiety symptoms (Clauss et al., 2014a; Jarcho et al., 2013a). While these differences in amygdala-prefrontal cortex neurocircuitry have been identified in adolescents and young adults with an inhibited temperament to date, no studies have examined temperament differences in brain activity in young children with an inhibited temperament.

Adults with an inhibited temperament have increased prefrontal cortex activation, including the anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (dlPFC), and dorsomedial prefrontal cortex (dmPFC) during both explicit and implicit emotion regulation tasks (Clauss et al., 2014a; Jarcho et al., 2013a, 2013b). The prefrontal cortex, including the anterior cingulate cortex (ACC), is

structurally connected to the amygdala (Carmichael and Price, 1995; Ghashghaei et al., 2007) and can function to inhibit amygdala response (Quirk et al., 2003). Greater activation of the prefrontal cortex may be an unsuccessful attempt at compensating for increased amygdala activity and emotional reactivity or it may be an effective method of suppressing emotional reactivity and may be protective against anxiety symptoms. Two studies have tested for the relationship between prefrontal cortex activity and anxiety symptoms in inhibited temperament and found that in adults with an inhibited temperament, increased prefrontal cortex activity (dlPFC and ACC) was associated with fewer anxiety symptoms (Clauss et al., 2014a; Jarcho et al., 2013a). These findings suggest that increased prefrontal cortex activity during cognitive control is an effective mechanism of suppressing emotional reactivity and may be associated with protection from or resilience to anxiety symptoms in inhibited individuals.

The prefrontal cortex has a number of functions, including cognitive control and emotion regulation, and prefrontal cortex function can be interrogated by different types of tasks. Some tasks require explicit emotion regulation, such as training subjects to reappraise their emotional responses, or using a Stroop task; other tasks tap implicit emotion regulation, such as having a subject anticipate and respond to an upcoming emotional stimulus without explicit instructions on how to regulate emotion. While explicit emotion regulation tasks ensure that subjects are engaging in a particular cognitive process, they may be less ecologically valid—rarely are individuals specifically instructed to engage in emotion regulation. In contrast, implicit emotion regulation occurs on a daily

basis, and differences in implicit emotion regulation can be easily tested in the scanner.

Implicit emotion regulation can be measured by testing for differences in brain activation during anticipation of emotional stimuli. Anticipatory processing can be adaptive, including planning and preparing for an upcoming situation, or it can be maladaptive, including anticipatory anxiety and physiological arousal, such as sweating, shaking, and nausea (Grupe and Nitschke, 2013). Particularly relevant for inhibited temperament, patients with social anxiety disorder often experience anticipatory anxiety, including a sense of fear, helplessness, and uncontrollable future threat (Barlow et al., 1996, pp. 251–328). In patients with social anxiety disorder, anticipatory anxiety leads to excessive worry and avoidance of otherwise safe situations. Patients with anxiety disorders commonly experience greater anticipatory anxiety for relatively minor events (Lorberbaum et al., 2004; Straube et al., 2007; Tillfors et al., 2002).

Given that anticipatory anxiety is a common feature of both the risk factor (inhibited temperament) and the psychiatric disorder (social anxiety disorder), the experimental study of neural activity during this key anticipatory period may provide a critical connection between brain function, the psychological process of anticipation, and the development of social anxiety in a high-risk group. We propose that identifying temperament differences in brain activation during anticipation will lead to an understanding of whether prefrontal cortex and cognitive control are altered in high-risk inhibited children prior to the development of anxiety disorders. Our working model is that high-risk children

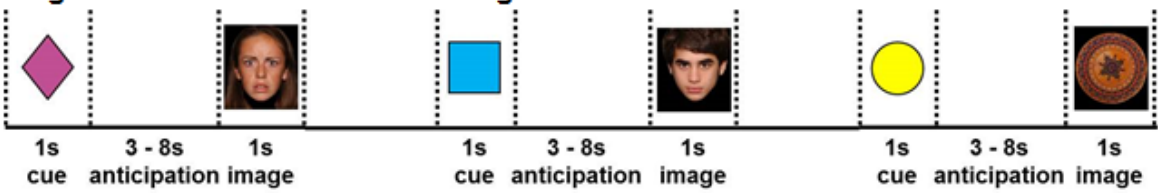
have dysfunction in both emotional reactivity brain regions (amygdala, BNST) and emotional regulation brain regions (dACC, dlPFC). We hypothesize that children with an inhibited temperament will have less activity in the prefrontal cortex (ACC, dlPFC, and dmPFC) and greater activity in the insula, BNST, and amygdala during anticipation of fear faces, relative to anticipation of neutral faces.

Methods

Study Protocol

Children were recruited using flyers, online recruitment databases and mass emails (see Chapter 2 for additional details about recruitment, screening, and phenotyping). Parents completed an online screening and eligible children and their parents came to the lab for three study visits. The first study visit included a clinical interview, behavioral assessment of temperament, and completion of child-report and parent-report questionnaires. The second study visit included an IQ test, mock MRI scan, and completion of any questionnaires not finished during the first study visit. The mock scan was completed to acclimate the child to the scanner and improve data quality (de Bie et al., 2010). Finally, the third study visit was the MRI scan. First, survey maps and a T1-weighted structural scan were collected. During this time, children watched a movie of their choice. Next, the resting state MRI scan was collected, and finally the anticipation task scan was collected.

Figure 7. Functional MRI task design



Experimental Design

FMRI Task. We used a cued anticipation task similar to ones previously used in a study of young adults with an inhibited temperament (Clauss et al., 2014a) and a number of studies in anxiety disorders (Aupperle et al., 2012; Brühl et al., 2013, 2011; Nitschke et al., 2009). The cued anticipation task included two event types, cue and image, and three conditions, fear face, neutral face, and neutral object. In an fMRI task, data are analyzed by comparing brain activation between a condition of interest and a baseline condition; we included the neutral object condition to be used as a baseline condition because neutral faces are not considered to be truly neutral by socially anxious individuals (Winton et al., 1995) and previous studies have found temperament differences in brain activation to neutral faces (Blackford et al., 2013, 2011, 2009; Pérez-Edgar et al., 2007; Schwartz et al., 2012).

Before the task, children completed a brief training to learn to associate a specific cue (colored shape) with a specific type of image (fear face, neutral face, neutral object). After the training, children were verbally tested to confirm they had learned the associations; two children (one inhibited, one uninhibited) who could not confirm the association between the cues and images repeated the training. The test phase of the task consisted of four runs. During each run,

children saw trials of a cue (1s; see Figure 7), followed by a brief anticipation period (jittered, 3-8s), and then an image (1s). Following each trial, there was a blank screen (jittered, 3-8s) before the next trial. The time between cue and image was jittered to provide an additional level of uncertainty. Each run consisted of eight trials of each condition (fear face, neutral face, neutral object) for a total of 24 trials per run and 32 of each trial condition across the entire task. The task was presented in Eprime (Version 2.0). We chose to use children's faces to increase ecological validity given that difficulties with peers (i.e. being excluded by them, being fearful of their judgment) are critical to the development of social anxiety disorder (Ollendick and Hirshfeld-Becker, 2002). Child emotional faces from the NIMH Child Emotional Faces Dataset (Egger et al., 2011) were used. Neutral objects were round, non-social objects the approximate size and shape of faces (i.e. a patterned bowl, a clock). Neutral objects came from several sources, including the IAPS image set (Lang et al., 1999), iStock Photo, and publically available images. To ensure attention to the task, children were asked to press one button whenever they saw a cue or an image.

MRI Acquisition. Data were collected using a 32-channel headcoil on a Philips 3 Tesla scanner. T1-weighted structural data were acquired using the following parameters: 256 mm FOV, 170 slices, 1 mm slice thickness, 0 mm gap. 2 s TR, 22 ms TE, 90° flip angle, 1.8 SENSE factor, 240 mm FOV, 3 × 3mm in plane resolution. Functional (EPI) data were acquired using the following parameters: 40 slices, 2.5mm slice thickness, 0.25m gap, and an axial oblique

acquisition, tilted 15 degrees, anterior higher than posterior, relative to the intercommisural plane.

MRI Preprocessing. Data were preprocessed using SPM8 (http://www.fil.ion.ucl.ac.uk/) implemented in Matlab 2010a (Version 7.10.0, Mathworks, Inc., Natick, MA). Preprocessing steps included: 1) slice time correction to the middle slice; 2) realignment of functional volumes to mean volume; 3) coregistration of functional and structural scans; 4) normalization of functional scans to EPI template (MNI template in SPM8); and 5) smoothing with a 6mm FWHM kernel. For each child, functional and structural data were visually inspected for artifacts, coverage, and signal dropout. One inhibited child was excluded for an artifact on the functional image. To restrict analyses to children who paid attention to the task, children were excluded for pressing the button less than 50% of the time for any stimulus type (two children, one inhibited and one uninhibited). Individual functional runs were excluded for: failing to press the button to at least 50% of stimuli in the run (3.3%), artifact on visual inspection (1.1%), or incomplete run in scanner (2.2%). This resulted in a final sample of 18 inhibited children and 19 uninhibited children with at least two usable functional runs.

Motion. Young children have difficulty staying still in the MRI scanner and motion may significantly affect data quality (Jezzard and Clare, 1999). There were no differences in motion between the two groups (maximum averaged across children $\pm$ standard deviation; translation: IT: 1.34 $\pm$ 1.1 mm; UT: 1.12 $\pm$ .91 mm; $p = .62$; rotation: IT: 0.024 $\pm$ 0.018 radians; UT: 0.017 $\pm$ 0.015 radians; p

= .30). To control for potential effects of motion, we used the robustly-weighted least squares toolbox (Diedrichsen and Shadmehr, 2005), which reduces the contribution of volumes with significant motion to the overall model.

Image Rating. To determine if the valence of images used in the study differed between inhibited or uninhibited children, following completion of the MRI, children rated the valence of the cues and of a subset of images presented in the study (10 images rated per stimulus type). Children were instructed to rate how "happy or sad the pictures made them feel" (1 = very happy; 5 = very sad). Image ratings were accompanied by schematics of each rating (i.e. the number one was presented above a smiley face). The cues and images were presented using Eprime software outside of the scanner environment. Valence rating data were missing for one uninhibited child.

Data Analysis

Behavioral Data. Button press data and image rating data were analyzed in SPSS (Version 21.0.0.0, IBM Corporation). Number of missed trials, reaction times, and valence ratings were analyzed using a repeated measures ANOVA with type (cue/image) and condition (fear face/neutral face/neutral object) as within-subject variables and temperament group as the between-subject variable.

FMRI Data Modeling. For each child, a general linear model was created in SPM8 with seven regressors: fear face cue, neutral face cue, neutral object cue, fear face image, neutral face image, neutral object image, and errors. To be consistent with other neuroimaging studies of anticipation, we modeled the cue

and image as our events of interest. Six contrasts were created for each subject: 1) anticipation of fear faces, relative to anticipation of neutral faces; 2) anticipation of fear faces, relative to anticipation of neutral objects; 3) anticipation of neutral faces, relative to anticipation of neutral objects; 4) fear face viewing, relative to neutral face viewing; 5) fear face viewing, relative to neutral object viewing; and 6) neutral face viewing, relative to neutral object viewing.

Statistical Analysis. Group differences in anticipatory processing and image viewing were tested using a two-sample t-test in SPM8. Activation to fear face and neutral face cues and images was compared to activation to neutral object cues and images.

Region of Interest Analysis. To reduce the overall number of comparisons, analyses were restricted to six anatomically-based regions of interest (ROIs). ROIs were based on findings in previous studies of anticipatory processing and inhibited temperament (Aupperle et al., 2012; Brühl et al., 2013, 2011; Clauss et al., 2014a; Jarcho et al., 2013a, 2013b; Nitschke et al., 2009). The ROIs were the amygdala, bed nucleus of the stria terminalis (BNST), insula, dorsolateral prefrontal cortex (dlPFC), medial prefrontal cortex (mPFC), and anterior cingulate cortex (ACC). The BNST mask was based on a recently published mask (Avery et al., 2014) and the insula, amygdala, and prefrontal cortex masks were based on the Automated Anatomical Labeling (Tzourio-Mazoyer et al., 2002) implemented in the Wake Forest Pick Atlas (Maldjian et al., 2003). Within each region, data were tested for group voxel-wise differences. Data were cluster-corrected ($\alpha < .05$) using AlphaSim

(http://afni.nimh.nih.gov/pub/dist/doc/manual/AlphaSim.pdf) with the following inputs: voxel-level $p < .05$, 3x3x3 mm voxel size, x=14.1, y=13.6, z=12.3 smoothness at FWHM, and 5,000 iterations. Temperament differences in activation were tested separately for each mask. Cluster thresholds were: amygdala (k = 21; 567 mL), BNST (k = 3; 81 mL), insula (k = 69; 1,863 mL), dlPFC (k = 164; 4,428 mL), dmPFC (k = 111; 2,997 mL), and ACC (k = 79; 2,133 mL).

Whole Brain Analysis. To identify other brain regions that differed between groups, but were not in our *a priori* hypothesized regions, a whole brain analysis was also performed. Cluster-based thresholding ($\alpha < .05$) in AlphaSim was used with the following inputs: voxel-level $p < .05$, 3x3x3 mm voxel size, calculated smoothness of the data at FWHM: x=14.1, y=13.6, z=12.3, and 5,000 iterations. Because the whole brain analysis was exploratory, we used a more conservative voxel p-value ($p < .005$) and the cluster threshold was k = 139 (3,753 mL) for a corrected $\alpha < .05$.

Post Hoc Analyses. To understand how brain activation changed over the course anticipation and image viewing, we conducted post hoc analyses based on significant temperament differences during anticipation. First, we created 6mm spheres around peak voxels in regions where there were significant group differences in activation during anticipation. Next, we extracted percent BOLD signal change from each of these spheres using EasyROI toolbox implemented in SPM8 (http://www.sbirc.ed.ac.uk/LCL/LCL_M1.html). We extracted percent signal change for the contrasts of fear face anticipation > neutral object

anticipation; fear face viewing > neutral object viewing; neutral face anticipation > neutral object anticipation; and neutral face viewing > neutral object viewing. Finally, we tested for group differences in signal change across both anticipation and image viewing using a two-sample t-test in SPSS.

Results

Behavioral Data

Missed Trials and Reaction Times. To see if the two groups differed on button press behavior, we tested for significant interactions of temperament (inhibited/uninhibited) with type (cue/image) and condition (fear face/neutral face/neutral object) on the number of missed button presses and on the button press reaction time. There was trend towards a main effect of type on reaction time ($p = .08$, $F = 3.28$, $df = 1$), with faster reaction times for images than cue (fear face cue: 571.5 + 86.2; neutral face cue: 568.9 + 86.2 ms; neutral object cue: 574.1 + 85.1; fear face image: 563.9 + 100; neutral face image: 572.1 + 88.4; neutral object image: 557.6 + 89.3). There were no other significant overall effects of type, condition, or interactions of type or condition with temperament on the percent of button presses missed or on the button press reaction times (all $p > .12$; see Table 7).

Table 7. Behavioral data

	IT		UT		
	Mean	SD	Mean	SD	p-value
Hit rate (%)					
Fear face cue	95.9	5.9	90.4	11.1	.07
Neutral face cue	92.0	11.0	91.6	8.8	.92
Neutral object cue	92.5	12.7	91.7	9.1	.84
Fear face image	94.7	6.5	91.3	8.9	.26
Neutral face image	94.7	7.6	93.6	6.5	.68
Neutral object image	92.9	9.2	90.8	8.5	.47
Reaction times (ms)					
Fear face cue	569.6	93.6	573.3	81.2	.90
Neutral face cue	560.4	91.3	576.8	67.9	.54
Neutral object cue	574.8	98.0	573.4	73.6	.96
Fear face image	561.6	118.8	566.1	81.7	.89
Neutral face image	569.5	108.3	574.6	67.4	.86
Neutral object image	550.6	96.1	564.2	84.5	.65
Valence ratings					
Fear face cue	2.4	1.2	2.2	1.2	.68
Neutral face cue	2.1	1.0	2.0	1.2	.77
Neutral object cue	1.9	0.9	2.4	1.1	.15
Fear face image	3.2	0.8	3.3	0.7	.58
Neutral face image	3.1	0.7	3.0	0.8	.84
Neutral object image	2.3	0.7	2.0	0.8	.29

Note: Children rated valence of the faces on a 1-5 Likert scale. Valence ratings ranged from 1 (very happy) to 5 (very unhappy). SD = standard deviation; ns = not significant.

Valence Ratings. To determine if the inhibited and uninhibited children interpreted the images differently, following the MRI scan, children were asked to

rate the valence of the cues and images. We tested for significant interactions between condition, type of stimulus, and temperament on valence rating data. There were significant interaction of type x condition x temperament ($p = .05$) and type x condition ($p \leq .001$) that qualified the main effects of main effects of type, condition, all $p \leq .001$; see Table 7). Based on these findings, we tested for an interaction of type x temperament within each condition. Within fear face condition, there was a main effect of type ($p < .001$, $F = 16.6$) but no interaction of temperament x type ($p = .50$, $F = 0.5$). Within the neutral face condition, there was a main effect of type ($p = .001$, $F = 16.2$), but no interaction of type x temperament ($p = .90$, $F < .1$). Finally, within the neutral object condition, there was no main effect of type, but there was a trend towards an interaction of type x temperament ($p = .08$, $F = 3.2$).

fMRI Data

FMRI contrasts were created for both cue conditions and image viewing conditions. First, to be consistent with a prior study of anticipation in inhibited temperament (Clauss et al., 2014a), we compared the fear face condition to the neutral face condition. Second, we compared both the fear face condition and the neutral face condition to the neutral object condition. Post hoc analyses were performed to unpack the initial findings. In post hoc analyses, we compared activation in regions with a temperament differences across cue and image.

Cue: Fear Face > Neutral Face. During anticipation of fear faces, relative to anticipation of neutral faces, the uninhibited children had greater activation in

two prefrontal regions, the rostral anterior cingulate cortex (rACC) and dorsomedial prefrontal cortex (dmPFC). Uninhibited children also had greater activation in the right insula, and bilateral amygdala (see Table 8 and Figure 8). There were no regions of temperament group difference on whole brain analysis. There were no regions where the inhibited group had greater activation for this contrast.

Cue: Fear Face > Neutral Object. During anticipation of fear faces, relative to anticipation of neutral objects, the uninhibited children had greater activation of a number of prefrontal cortex regions, including the rACC, right dorsolateral prefrontal cortex (dlPFC), and dmPFC (all $p < .05$, corrected; see Table 8 and Figure 10). On whole brain analysis, the uninhibited group had greater activation in a region of the cerebellum ($p < .05$, corrected; see Table 8). There were no regions where the inhibited group had greater activation for this contrast.

Table 8. Temperament differences in brain activation by functional MRI.

Brain Region (Hemisphere)	Brodmann Area	Cluster Size (voxels)	Z Score	Peak Voxel x	y	z
Fear Face Cue > Neutral Face Cue						
Uninhibited Temperament > Inhibited Temperament						
Region of interest analysis						
Anterior cingulate cortex (B)	24, 32	241	3.15	-3	47	-2
Insula (R)	13, 47	76	2.72	33	8	-11
Dorsomedial prefrontal cortex (B)	10	374	3.71	0	62	22
Amygdala (R)	NA	32	2.62	27	-7	-11
Amygdala (L)	NA	28	2.28	-30	2	-20
Fear Face Cue > Neutral Object Cue						
Uninhibited Temperament > Inhibited Temperament						
Region of interest analysis						
Rostral anterior cingulate cortex (B)	10, 24, 32	295	2.86	6	44	16
Dorsolateral prefrontal cortex (R)	8, 9, 10	181	2.61	27	41	46
Dorsomedial prefrontal cortex (B)	9, 10, 24	321	3.99	3	71	4
Whole brain analysis						
Cerebellum (L)	NA	162	4.17	-45	-61	-47
Neutral Face Cue > Neutral Object Cue						
Inhibited Temperament > Uninhibited Temperament						
Region of interest analysis						
Insula (R)	13	150	3.36	33	-19	13
Insula (L)	13	90	2.61	-30	-19	19
Whole brain analysis						
Visual cortex (B)	17, 18	305	3.65	3	-97	10
Insula (L)	44, 45, 46	161	3.46	-39	29	22
Fear Face Image > Neutral Face Image						
Inhibited Temperament > Uninhibited Temperament						
Region of interest analysis						
Dorsomedial prefrontal cortex (B)	10	105	3.22	0	65	22
Fear Face Image > Neutral Object Image						
Inhibited Temperament > Uninhibited Temperament						
Region of interest analysis						
Anterior cingulate cortex (B)	10, 32	314	4.41	15	44	22
Dorsolateral prefrontal cortex (L)	10, 46	487	3.98	-15	65	4
Dorsolateral prefrontal cortex (L)	8, 9	184	3.26	-15	41	37
Dorsolateral prefrontal cortex (R)	8, 9, 10	931	4.30	18	44	22
Dorsomedial prefrontal cortex (B)	8, 9, 10	900	4.17	15	41	22
Whole brain analysis						
Visual cortex, precuneus (B)	18, 19, 30	1234	4.17	-27	-70	-14
Medial prefrontal cortex (B)	9, 10, 32	696	4.41	15	44	22
Dorsolateral prefrontal cortex (R)	45, 46	245	3.69	54	32	13
Neutral Face Image > Neutral Object Image						
Inhibited Temperament > Uninhibited Temperament						
Region of interest analysis						
Insula (R)	13	101	3.65	33	-31	19
Dorsolateral prefrontal cortex (R)	8, 9, 10	889	3.58	15	44	46
Dorsolateral prefrontal cortex (L)	10	223	3.27	-36	47	1
Dorsomedial prefrontal cortex (B)	8, 9, 10	671	3.53	9	38	46
Whole brain analysis						
Dorsal medial prefrontal cortex (B)	8	117	3.58	15	44	46
Dorsolateral prefrontal cortex (R)	9, 45, 46	201	3.59	60	17	25
Visual cortex, precuneus (R)	7, 31	800	3.87	33	-34	19
Lateral prefrontal cortex (L)	10	144	3.27	-36	47	1
Cerebellum (R)		155	3.59	27	-70	-41

Note: B = both; R = right; L = left; coordinates are in MNI space.

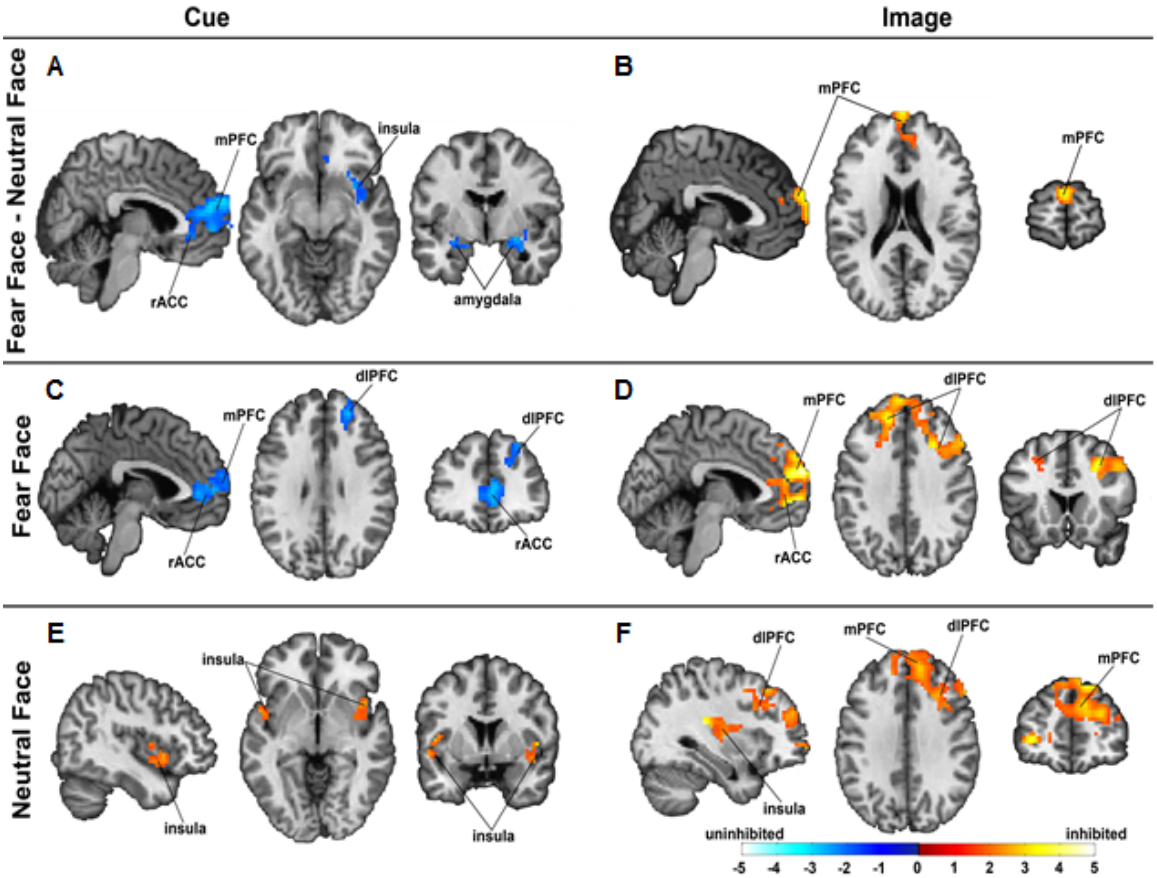
Cue: Neutral Face > Neutral Object. During anticipation of neutral faces,

relative to anticipation of neutral objects, the inhibited group had greater activation of the bilateral insula (all $p < .05$, corrected; see Table 8 and Figure 10). On whole brain analysis, the inhibited group had greater activation in the primary visual cortex and left insula (all $p < .05$, corrected; see Table 8 and Figure 10). There were no regions where the uninhibited group had greater activity.

Image: Fear Face > Neutral Face. When viewing fear faces, relative to viewing neutral faces, the inhibited group had greater activation of the dmPFC ($p < .05$ corrected; see Table 8 and Figure 10). There were no regions where the uninhibited group had greater activation.

Image: Fear Face > Neutral Object. When viewing fear faces, relative to viewing neutral objects, the inhibited group had greater activation of the anterior cingulate cortex, bilateral dorsolateral prefrontal cortex, and dorsomedial prefrontal cortex (all $p < .05$, corrected; see Table 8 and Figure 10). On whole brain analysis, the inhibited group had greater activation of the visual cortex, precuneus, and medial and right dorsolateral prefrontal cortex (all $p < .05$, corrected; see Table 8 and Figure 8). There were no regions where the uninhibited group had greater activation.

Figure 8. Differences in functional activation across the MRI task.



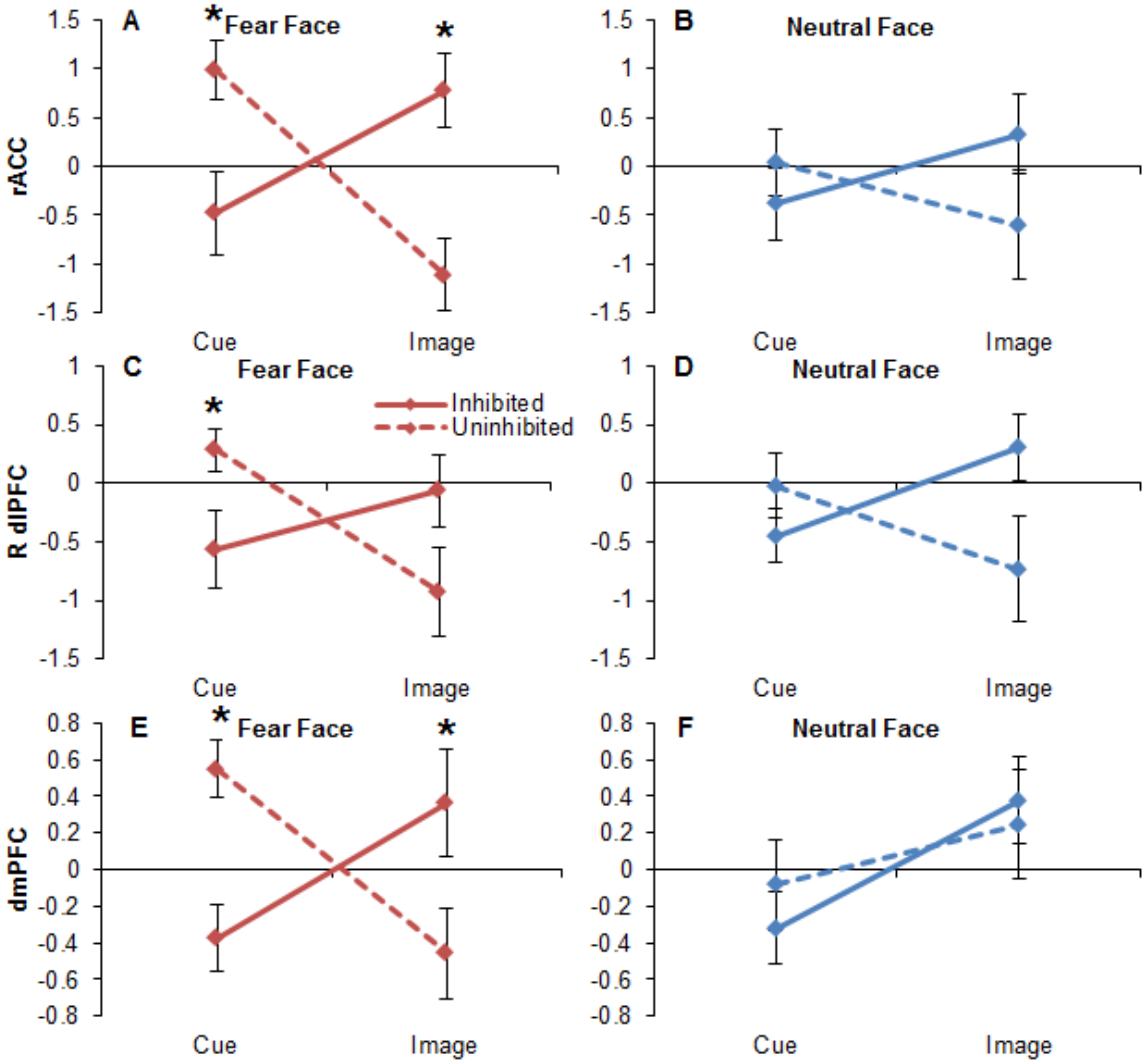
A) During anticipation of fear faces, relative to anticipation of neutral faces, inhibited subjects have less activation in the medial prefrontal cortex (mPFC), rostral anterior cingulate cortex (rACC), right insula, and bilateral amygdala, compared to uninhibited subjects (all $p < .05$). **B)** While viewing fear faces, relative to viewing neutral faces, inhibited subjects had greater activation of the medial prefrontal cortex, than the uninhibited subjects. **C)** During anticipation of fear faces, relative to anticipation of neutral faces, inhibited subjects had less activation of the mPFC, rACC, and right dorsolateral prefrontal cortex (dlPFC). **D)** While viewing fear faces, inhibited subjects had greater activation of the mPFC, rACC, and bilateral dlPFC. **E)** During anticipation of neutral faces, inhibited subjects had greater activation of the bilateral insula. **F)** When viewing neutral faces, inhibited subjects had greater activation of the mPFC, bilateral dlPFC, and right insula.

Image: Neutral Face > Neutral Object. When viewing neutral faces, relative to viewing neutral objects, the inhibited group had greater activation of the right insula, bilateral dorsolateral prefrontal cortex, and dorsomedial prefrontal cortex (all $p < .05$, corrected; see Table 8 and Figure 10). On whole brain analysis, the inhibited group had greater activity of the medial prefrontal

cortex, lateral prefrontal cortex, and right dorsolateral prefrontal cortex, visual cortex, and cerebellum (all $p < .05$, corrected; see Table 8 and Figure 8). There were no regions where the uninhibited group had greater activity.

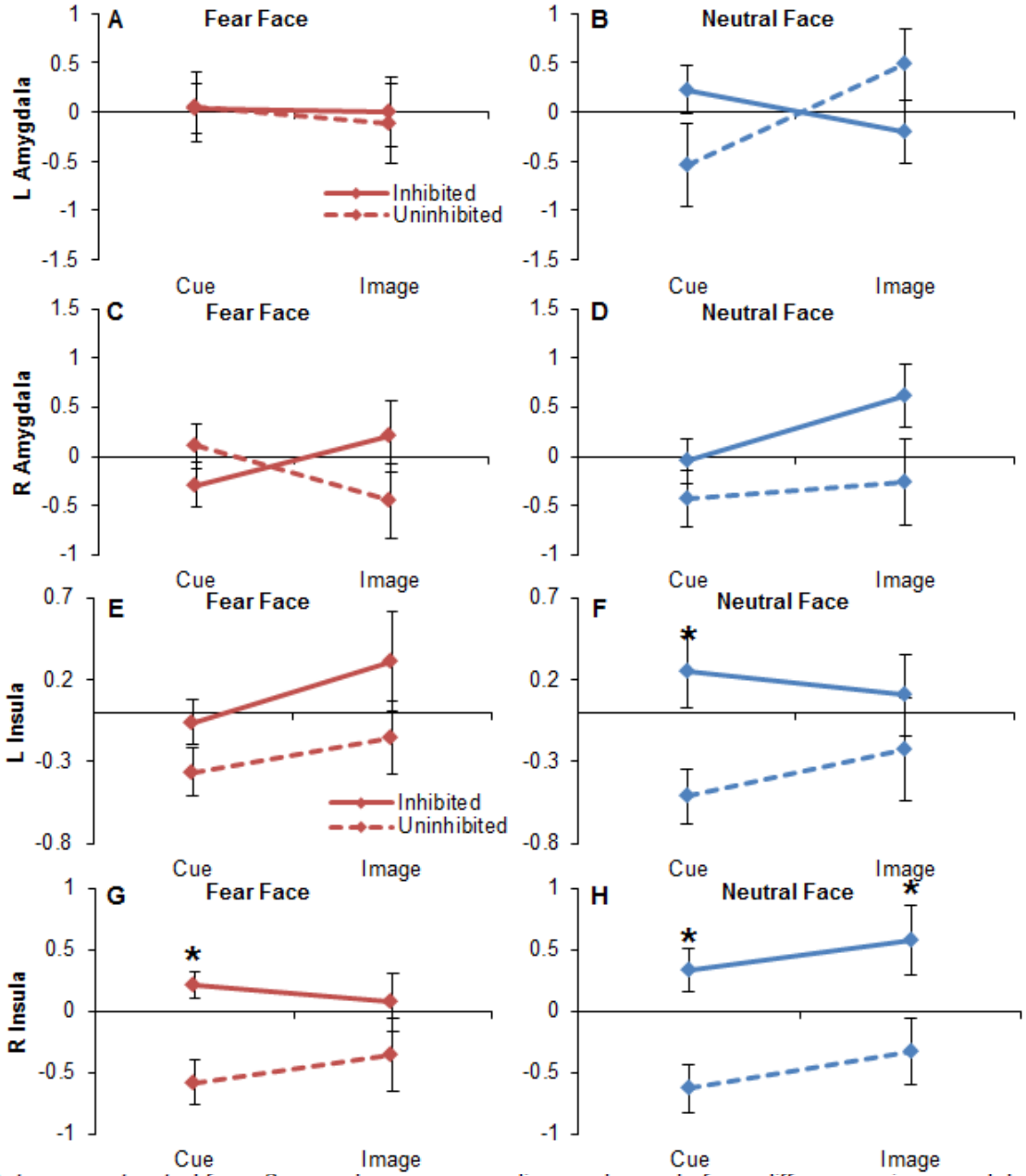
Post Hoc Analyses. To unpack the neuroimaging findings, spheres were created around the peak voxels of the regions of difference during anticipation: rostral anterior cingulate cortex (center: 6, 44, 16); right dorsolateral prefrontal cortex (center: 27, 41, 46); dorsomedial prefrontal cortex (center: 3, 71, 4); left amygdala (center: -30, 2, -20); right amygdala (center: 27, -7, -11); left insula (center: -30, -19, 19); and right insula (center: 33, -19, 13). We tested for group differences in brain activity during anticipation and image viewing. In the prefrontal cortex, inhibited children had significantly less activation during fear face anticipation in the rACC, right dlPFC, and dmPFC, but greater activation during image viewing (see Figure 9). There were no significant differences in neutral face anticipation and viewing in the rACC, right dlPFC, or dmPFC. There were no significant differences in amygdala activation during anticipation or viewing of faces relative to objects (see Figure 10 A-D). In the left insula, the inhibited group had significantly greater activity to neutral face anticipation, but no significant differences to fear face anticipation or viewing or neutral face viewing (see Figure 10 E-H). In the right insula, the inhibited group had significantly greater insula activation during fear and neutral face anticipation and during neutral face viewing.

Figure 9. Activation of the prefrontal cortex across cue and image.



Data was extracted from 6mm spheres surrounding peak voxels from differences in prefrontal cortex activation during anticipation. **A)** Inhibited subjects had significantly less rACC activation during anticipation of fear faces, relative to anticipation of neutral faces, but had significantly greater activation during fear face viewing, compared to neutral face viewing, relative to the uninhibited temperament group ($p < .05$). **B)** There were no differences in rACC activation to neutral faces. **C)** The inhibited temperament group had significantly less right dlPFC activation during anticipation of fear faces, relative to neutral objects. There were no differences in right dlPFC activity to faces. **D)** There were no significant temperament differences in right dlPFC activity during anticipation or viewing of fear faces. **E)** The inhibited temperament group had less activation of the dmPFC during anticipation of fear faces, relative to anticipation of neutral objects, and greater activation of the dmPFC when viewing fear faces, relative to neutral objects, compared to the uninhibited temperament group. **F)** There were no temperament differences in dmPFC activity during anticipation and viewing of neutral faces. * denotes $p < .05$

Figure 10. Activation of the amygdala and insula across cue and image.



Data was extracted from 6mm spheres surrounding peak voxels from differences in amygdala activation during fear face anticipation, relative to neutral face anticipation and from differences in insula activation during neutral face anticipation, relative to neutral object anticipation. **A)** There were no significant differences in left amygdala activation during fear face anticipation or viewing and **B)** no differences in left amygdala activation during neutral face anticipation or viewing. **C)** There were no significant differences in right amygdala activation during fear face anticipation or viewing and **D)** neutral face anticipation or viewing. **E)** There were no significant differences in left insula activation during fear face anticipation or viewing and **F)** inhibited subjects had significantly greater left insula activation during neutral face anticipation, but not during neutral face viewing. **G)** Inhibited subjects had significantly greater right insula activation during anticipation of fear faces, but not during fear face viewing and **H)** inhibited subjects had significantly greater activation during neutral face anticipation and viewing. * denotes $p < .05$

Discussion

This study provides the first evidence that inhibited children have less prefrontal regulatory activity during anticipation of fear faces, a process that engages intrinsic cognitive control. Children with an inhibited temperament are at increased risk for developing social anxiety disorder, a disorder associated with anticipatory anxiety in social situations. The inhibited children also had increased rACC, mPFC, and dlPFC activity when viewing fear faces, suggesting that inhibited children may have a delayed regulatory response or may fail to effectively prepare for negative social stimuli.

During anticipation of viewing fear faces, inhibited children had a deficit in prefrontal cortex activation, specifically within the rACC, dlPFC, and dmPFC. These deficits likely represent a neural risk factor for social anxiety disorder—reduced function of cognitive control regions during a task that engages implicit emotion regulation. These findings are consistent with studies of adults with anxiety disorders that found decreased ACC activity during tasks which require emotion regulation (Blair et al., 2012; Britton et al., 2013; Goldin et al., 2009a, 2009b; Swartz et al., 2014), and studies that show that treatments for anxiety, such as cognitive behavior therapy, increase activation of the prefrontal cortex, including medial prefrontal cortex and dlPFC, during emotion regulation (Goldin et al., 2013).

The prefrontal cortex undergoes protracted development from childhood, through adolescence, and into young adulthood, as measured by gray matter density, synaptogenesis, and myelination (Casey et al., 2000; Gogtay et al.,

2004; Huttenlocher, 2002). Development of the prefrontal cortex parallels the development of cognitive control in children (Bunge et al., 2002; Pitskel et al., 2011). For inhibited children, developmental trajectories likely diverge as the prefrontal cortex continues to develop. In children, cognitive control is associated with more dmPFC activity and less amygdala activity (Pitskel et al., 2011), additionally, increased dlPFC activity correlates with better cognitive control (Bunge et al., 2002). Some children may develop stronger emotion regulation skills and develop the ability to engage the prefrontal cortex during anticipation; we predict that these children will go on to be resilient and not develop anxiety disorders. Other children may continue to fail to engage prefrontal cortex during anticipation of fear faces, or more importantly, during anticipation of negative social situations; we predict that these children will be more susceptible to anxiety disorders. To understand these questions, longitudinal studies are needed to follow at-risk, inhibited children until they reach adolescence and young adulthood. Longitudinal studies will tell us if prefrontal cortex activity is a predictor of the development of anxiety disorders.

Across the paradigm, the inhibited group had a hyperactive insula response, with increased insula response to anticipation of fear faces and neutral faces, and increased insula response during neutral face viewing (see Figure 12E-H). The insula is implicated in interceptive processing, such as feelings of heart racing, sweating, and nausea. Paulus and Stein (2006) proposed “an insular view of anxiety,” the posterior insula receives information from the periphery about visceral sensations and these sensations are integrated into in

the anterior insula. The anterior cingulate generates predictions about upcoming body states or outcomes of future events, particularly for aversive events, and this information feeds back to the anterior insula. Patients with anxiety disorders overestimate the likelihood that they will feel anxiety symptoms in the future, and thus experience anticipatory anxiety. During anticipation of negative stimuli, the insula is activated in healthy controls (Carlson et al., 2011; Chua et al., 1999; Ploghaus et al., 1999; Sarinopoulos et al., 2010; Simmons et al., 2004), individuals with high trait anxiety have more insula activation during anticipation (Clauss et al., 2014a; Drabant et al., 2010; Simmons et al., 2011, 2008, 2006), and finally, patients with anxiety disorders have increased insula activation during anticipation (Aupperle et al., 2012; Simmons et al., 2013; Straube et al., 2007). Anticipatory insula activity can be reduced with common treatments for anxiety disorders, including selective serotonin reuptake inhibitors (Aupperle et al., 2011; Simmons et al., 2009), suggesting that insula activity may be a target for intervention in preventing or reducing anxiety symptoms.

We hypothesized that inhibited children would have greater amygdala activation during anticipation of fear faces, given prior findings of increased amygdala activation during anticipation of negative stimuli in anxiety disorders (Brühl et al., 2011; Nitschke et al., 2009; Straube et al., 2007) and findings of amygdala hyperactivity in inhibited temperament (Blackford et al., 2011, 2009; Clauss et al., 2011; Pérez-Edgar et al., 2007; Schwartz et al., 2003a). We failed to find support for this hypothesis. Instead, inhibited children had a similar amygdala response during both anticipation of fear faces and anticipation of

neutral faces (see Figure 10A). These findings are consistent with prior studies of inhibited adults showing similar amygdala activation to both novel and familiar faces (Blackford et al., 2011) and when passively viewing different types of emotional faces (Pérez-Edgar et al., 2007). In contrast, the uninhibited group showed the pattern commonly found in healthy controls, increased amygdala activation during anticipation of fear faces. When amygdala activation during anticipation of faces was compared to anticipation of neutral objects, there were no significant differences between the groups (see Figure 12 A-D), suggesting that the differences in amygdala activation are due to heightened amygdala activation to neutral faces in the inhibited group. Patients with social anxiety disorder have greater amygdala activation to neutral faces (Cooney et al., 2006), and inhibited adults rated neutral faces as being more negative than the uninhibited group (Clauss et al., 2014a). Individuals with high social anxiety symptoms also consider neutral faces to be more negative (Winton et al., 1995). Inhibited children had less differential amygdala activation to anticipation of fear faces, relative to anticipation of neutral faces, suggesting that they engage anticipatory amygdala reactivity to faces in general. Increased amygdala reactivity to faces in general may be a mechanism by which inhibited temperament confers risk for social anxiety disorder.

No significant temperament differences in bed nucleus of the stria terminalis (BNST) activity were identified. The BNST is a small, subcortical region which responds to sustained anxiety and uncertain threat (Alvarez et al., 2011; Davis, 1998; Somerville et al., 2012, 2010; Walker and Davis, 2008).

Additionally, we may not have identified differences in BNST activity because our anticipation period was relatively short (3 - 8 seconds) and other neuroimaging studies that identified BNST activity used longer time periods. Future studies using sequences optimized for the activation and detection of BNST activity will be critical to test if this is a neural substrate of inhibited temperament and anticipatory processing.

This study had a number of limitations. First, children were selected based on being high or low on inhibited temperament, and thus did not represent the full range of temperament. However, previous studies have shown that the uninhibited temperament group has similar patterns to adults with an average temperament (Blackford et al., 2013). Second, children in this study had relatively high IQs (mean: 116, see Chapter 2), which may make the results less generalizable; however, high IQs are common in neuroimaging studies of children, and so our results can be compared to other neuroimaging studies of children (Blair et al., 2011; Guyer et al., 2012, 2006). Finally, online anxiety ratings were not collected because cognitive tasks, such as rating anxiety levels, have been shown to alter brain response (Pérez-Edgar et al., 2007).

Conclusions

In conclusion, inhibited children have less activity in a prefrontal network, including the rostral anterior cingulate cortex, dorsomedial prefrontal cortex, and dorsolateral prefrontal cortex during the anticipation of fear faces. Inhibited children may fail to effectively prepare for social stimuli, and attempt to

compensate for that by activating prefrontal cortex when viewing social stimuli. Future studies should continue to study inhibited children prior to the development of anxiety disorders and track them over time, to determine if anticipatory prefrontal cortex activation predicts the development of social anxiety disorder.

CHAPTER IV

ALTERATIONS IN AMYGDALA-DORSAL ANTERIOR CINGULATE CORTEX FUNCTIONAL CONNECTIVITY

Introduction

Patients with complex psychiatric disorders, such as social anxiety, have underlying alterations in brain activation and brain connectivity (Brühl et al., 2013, 2011; Dodhia et al., 2014; Etkin and Wager, 2007; Freitas-Ferrari et al., 2010; Goldin et al., 2009b; Hattingh et al., 2013; Klumpp et al., 2013a, 2013b; Labuschagne et al., 2011; Shah et al., 2009). Specifically, anxiety disorders have been consistently associated with less connectivity between the amygdala, a region involved in responding to salient stimuli and generating fear responses, and the prefrontal cortex, a region involved in regulating emotions and suppressing amygdala activity. Functional connectivity measures the correlation between activity in two brain regions (Friston et al., 1997), and less functional connectivity implies that two regions are less able to communicate or functionally influence each other. The prefrontal cortex can inhibit amygdala response (Quirk et al., 2003). Children and adults with anxiety disorders have less structural and functional connectivity between the amygdala and the prefrontal cortex (Baur et al., 2013, 2011; Birn et al., 2014; Phan et al., 2009; Prater et al., 2013; Roy et al., 2013). Reduced amygdala-prefrontal cortex connectivity is also present in non-human primates with an inhibited temperament (Birn et al., 2014), adults with an

inhibited temperament (Blackford et al., 2014), and individuals at increased genetic risk for anxiety disorders (Pezawas et al., 2005).

Examining prefrontal cortex connectivity may help to interpret conflicting findings of increased and decreased prefrontal cortex activation in inhibited temperament. Individuals with an inhibited temperament have less prefrontal cortex activation when reacting to faces (Clauss et al., 2011). In contrast, inhibited temperament is also associated with increased prefrontal cortex activation during tasks that engage cognitive control or emotion regulation, such as the Stroop task or a cued anticipation task (Clauss et al., 2014a; Jarcho et al., 2013a, 2013b). These findings can be explained by several hypotheses: 1) individuals with an inhibited temperament may have more activation of the prefrontal cortex during cognitive control to compensate for amygdala hyperactivity; 2) individuals with an inhibited temperament have weaker functional connectivity between the amygdala and prefrontal cortex; or 3) individuals with an inhibited temperament have a combination of both amygdala hyperactivity and weaker amygdala-PFC connectivity.

To date, three studies have tested for differences in amygdala-prefrontal connectivity in adults with an inhibited temperament. First, during an attention bias task, adults with a history of inhibited temperament had more negative connectivity between the amygdala and the dorsolateral prefrontal cortex (dlPFC; Hardee et al., 2013). While the amygdala and dlPFC are not structurally connected, the dlPFC projects to the dorsal anterior cingulate cortex (dACC; Barbas and Pandya, 1989), which projects to both the rostral anterior cingulate

cortex (rACC) and the amygdala (Carmichael and Price, 1995; Ghashghaei et al., 2007). Second, at rest, adults with inhibited temperament had less positive connectivity between the amygdala and prefrontal regions, including the dorsal anterior cingulate cortex (dACC; Blackford et al., 2014). Third, adults with an inhibited temperament had more negative connectivity between the amygdala and the rACC, and more positive connectivity between the dACC and the dlPFC during anticipation of viewing fear faces (Clauss et al., 2014a). In inhibited temperament, connectivity in an amygdala-ACC-dlPFC circuit is altered; the dACC may be the critical node in this circuit.

The dACC (also known as the anterior mid-cingulate cortex) has a number of functions, including cognitive control, negative affect, and pain (Bush et al., 2000; Shackman et al., 2011) and has reciprocal structural connections with the basolateral and basomedial subregions of the amygdala (Ghashghaei et al., 2007). Connectivity between the amygdala and medial prefrontal cortex, including the dACC, is critical for emotion regulation (Etkin et al., 2011). Increased dACC activation and decreased amygdala activation is associated with more successful emotion regulation (Phan et al., 2005), and increased dACC thickness is associated with more frequent use of emotion regulation strategies in healthy adults (Giuliani et al., 2011). While a number of other brain regions, such as the dlPFC and dorsomedial prefrontal cortex (dmPFC), are implicated in emotion regulation, the study of differences in amygdala connectivity with the dACC may be most useful, as many of the other regions activated during emotion regulation lack direct structural connections with the amygdala

(Ghashghaei et al., 2007; Morecraft and Van Hoesen, 1998; Morecraft et al., 2007). In this study, we propose to test for temperament differences in amygdala-dACC connectivity.

Differences in amygdala-dACC connectivity can be tested for using multiple methods, including during a task or during a state of rest. Task-based connectivity tests for differences in connectivity over the course of the task, which can be useful in understanding how connectivity changes during different conditions. Resting state connectivity provides a measure of intrinsic connectivity networks. Resting state networks are a useful measure of brain connectivity because: 1) the vast majority of the brain's metabolic processes are active at rest (Raichle and Mintun, 2006); 2) resting state networks are stable across subjects and time points (Biswal et al., 2010); 3) resting state networks are present in different states of consciousness (Hutchison et al., 2013); and 4) resting state networks are replicable across species (Birn et al., 2014; Oler et al., 2012). Mounting evidence shows that resting state functional MRI recruits a variety of functional networks that can be engaged during tasks and may represent underlying structural connectivity (Buckner et al., 2013; Shen et al., 2012).

In this study, we tested for temperament differences in amygdala-dACC connectivity. To assess group differences in connectivity, we measured functional connectivity during an emotional task (anticipation and viewing of fear faces) and at rest. Additionally, we compared amygdala-dACC connectivity within subjects between the emotional task and resting state, to determine if connectivity during the social anticipation task is based on underlying resting

state connectivity. Based on findings in adults with an inhibited temperament, we hypothesized that during anticipation of fear faces and at rest, inhibited children would have less amygdala-dACC connectivity, and that these two connectivity measures would be correlated.

Methods

Recruitment

Study participants were 40 children (20 inhibited, 20 uninhibited) who had participated in a study of temperament. Children were recruited for: 1) being between the ages of 8-10 years; 2) having no contraindications for MRI; and 3) having no history of psychiatric diagnosis or treatment (see Chapter 2 for details on recruitment and screening). There were no differences between temperament groups in age, gender, ethnicity, or handedness (see Table 3).

Experimental Design

Two types of functional connectivity data were collected. Task-based functional connectivity data were collected from a social anticipation task and preprocessed as detailed in Chapter 3. Resting state connectivity data were collected as detailed below prior to collection of the task-based data.

Task-Based Connectivity Data

To identify temperament differences in functional connectivity between the

amygdala and the dACC, we used psychophysiological interaction analysis (PPI; Friston et al., 1997). For the amygdala seed region, we used an amygdala mask from the AAL atlas of the Montreal Neurological Institute (Tzourio-Mazoyer et al., 2002). For the dACC, we used a 6mm radius sphere around coordinates previously associated with anticipation of fear faces in adults with an inhibited temperament (x = -12, y = 33, z = 27; Clauss et al., 2014a). Using the generalized psychophysiological interaction toolbox (gPPI; http://www.brainmap.wisc.edu/PPI), average BOLD time series were extracted from each seed region. PPI analysis uses three regressors: the physiological regressor, the psychological regressor, and the interaction regressor. The physiological vector was the BOLD time series, and the psychological vector was one of the following contrasts: 1) anticipation of fear faces, relative to anticipation of neutral faces; 2) anticipation of fear faces, relative to anticipation of neutral objects; 3) anticipation of neutral faces, relative to anticipation of neutral objects; 4) fear face viewing, relative to neutral face viewing; 5) fear face viewing, relative to neutral object viewing; and 6) neutral face viewing, relative to neutral object viewing. The interaction regressor modeled the change in amygdala connectivity between the conditions in the contrast. The regressors were used to model the BOLD time series in individual subjects, producing an estimate of differences in connectivity at each voxel. The resulting contrast images were used to test for differences in connectivity.

Resting State Functional Connectivity Data

Data Acquisition. Scans were collected using a 32-channel headcoil on a 3 Tesla Philips scanner (Philips Healthcare, Inc, Best, The Netherlands). Seven minutes of functional MRI "resting state" data were obtained approximately 15 minutes after entering the scanner, following structural MRI data collection. Children were instructed to "close their eyes and relax, but try not to fall asleep". High resolution T1-weighted structural images were collected using the following parameters: 256 mm FOV, 170 slices, 1 mm slice thickness, 0 mm gap. Functional images were acquired using the following parameters: 2s TR; 1.8 SENSE; 240 mm FOV; 3 x 3 mm in plane resolution using an 80 x 80 matrix (reconstructed to 128 x 128). Each functional volume contained 28 4 mm slices (acquisition voxels = 3 mm x 3 mm x 4 mm) and provided whole brain coverage.

Data Processing. Resting state functional data were preprocessed in SPM8. Each participant's T1W structural image was segmented into gray matter, white matter, and cerebrospinal fluid using standard segmentation in SPM8. Next, data was slice time corrected, motion corrected, coregistered to the segmented gray matter image, and normalized using parameters from normalization of the gray matter image. Images were resampled to 3 x 3 x 3 mm voxels and spatially smoothed (6 mm at full width half maximum). There were no significant differences between the two groups in motion (translation: IT: 0.82 $\pm$ 0.85 mm; UT: 0.98 $\pm$ 1.12 mm; $p = .63$; rotation: IT: 0.015 $\pm$ 0.014 radians; UT: 0.017 $\pm$ 0.025 radians; $p = .76$). Because motion can affect resting state connectivity (Power et al., 2012), we censored any volumes with greater than

2mm motion in any direction and any volumes with high signal intensity (Z scores > 9) using the Artifact Repair Toolbox (Mazaika et al., 2007).

Intrinsic Connectivity. Intrinsic resting state connectivity was calculated using the CONN toolbox (Whitfield-Gabrieli and Nieto-Castanon, 2012). The amygdala was used as the seed region. The amygdala seed region was from the AAL atlas of the Montreal Neurological Institute (Tzourio-Mazoyer et al., 2002). The BOLD time series for each subject was estimated as the average time series for all voxels in each ROI. To remove potential sources of noise, BOLD signal was band pass filtered (.01 to .1 Hz), and white matter and CSF signals were removed. Using the artifact detection toolbox (ART; http://www.nitrc.org/projects/artifact_detect/), volumes with greater than 2mm of motion or a global mean standard deviation greater than Z = 9 were removed. Temporal correlations were estimated between the seed region ROI time course and every other voxel in the brain, which produced a beta image for each subject and each seed region ROI. The resulting beta images were used for all subsequent analyses.

Data Analysis

Amygdala-dACC Connectivity. To test for differences in amygdala-dACC connectivity during the task and at rest, beta values of connectivity were extracted using the EasyROI tool (http://www.sbirc.ed.ac.uk/LCL/LCL_M1.html). We tested for temperament differences in task-based and resting state

connectivity between the two groups using a two-sample t-test in SPSS (version 21.0.0).

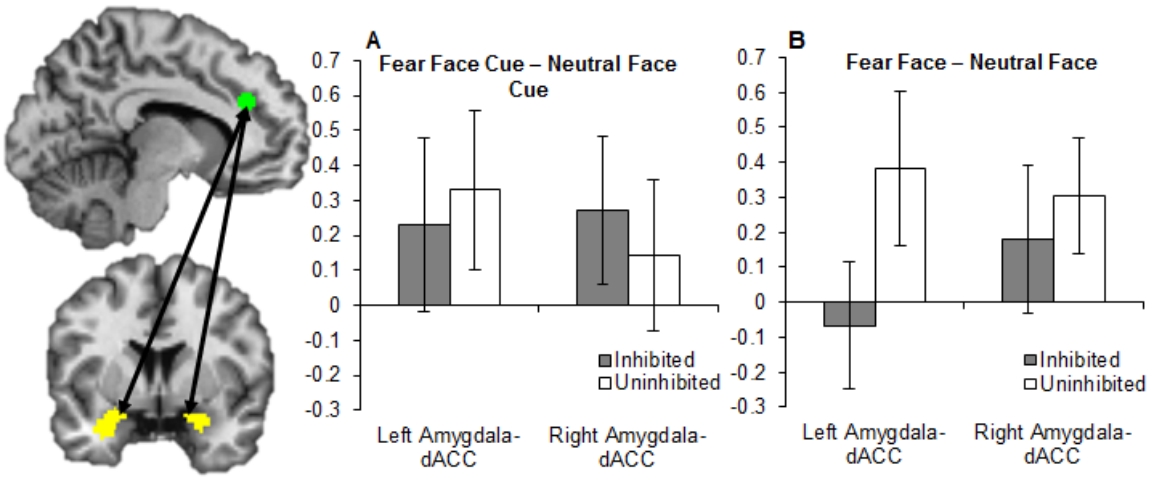
Comparisons between Task and Resting State Connectivity. To determine if functional connectivity during an emotional task is related to differences in resting state connectivity, we compared amygdala-dACC connectivity between the anticipation of fear faces and fear face viewing to resting state using three methods: 1) to determine if the relationship between task and resting state connectivity differed between temperament groups, we conducted a repeated measures analysis of variance (ANOVA) with task (emotional/rest) as the within-subjects factor and group (inhibited/uninhibited) as the between-subjects factor 2) to determine if there was an overall relationship between task and resting state connectivity, we tested for a correlation between emotional and non-emotional connectivity values across all children, and 3) to determine if there was a relationship between task and resting state connectivity within each group, we tested for a correlation between emotional and non-emotional connectivity values within each temperament group.

Results

Task-Based Functional Connectivity

There were no significant differences between groups in amygdala-dACC connectivity during fear face anticipation, relative to neutral face anticipation or

Figure 11. Amygdala-dACC connectivity during fear face anticipation and viewing.



There were no significant temperament differences in amygdala-dACC connectivity across **A)** anticipation of fear faces and **B)** viewing fear face (all p > .13).

during fear face viewing, relative to neutral face viewing (all p > .13, see Figure 11 and Table 9).

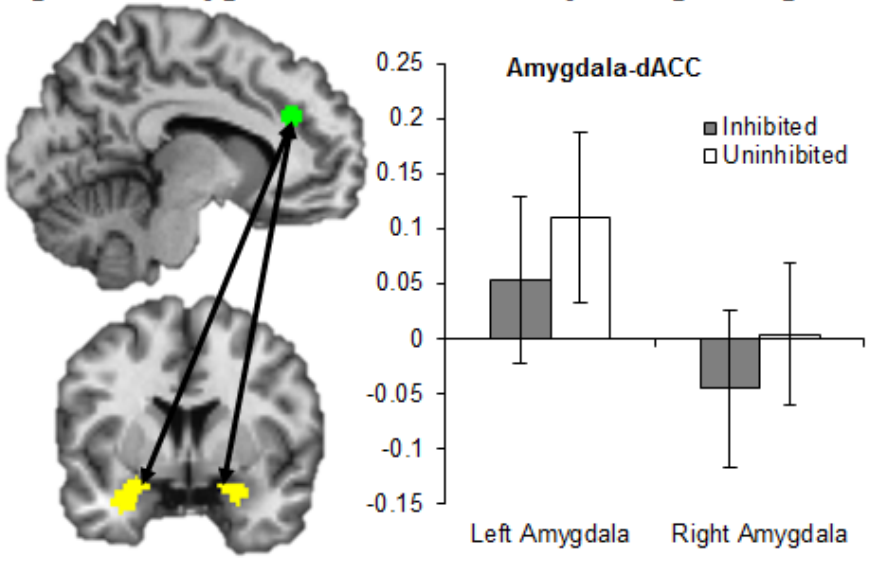
Resting State Connectivity

There were no significant differences in amygdala-dACC resting state connectivity between temperament groups (all p > .33, see Figure 12 and Table 9).

Relationship between Task-Based and Resting State Connectivity

To test for a relationship between task-based and resting state connectivity, we used three methods. First, to test if the relationship between task-based and resting state connectivity differed between groups, we conducted a repeated measures ANOVA. There was no significant interaction of connectivity type by temperament in the left (p = .78) or right amygdala (p = .69). Second, to test for an overall relationship between task-based and functional

Figure 12. Amygdala-dACC connectivity during resting state.



There were no significant differences in amygdala-dACC connectivity during resting state ($p > .60$).

Table 9. Temperament differences in dACC-amygdala connectivity.

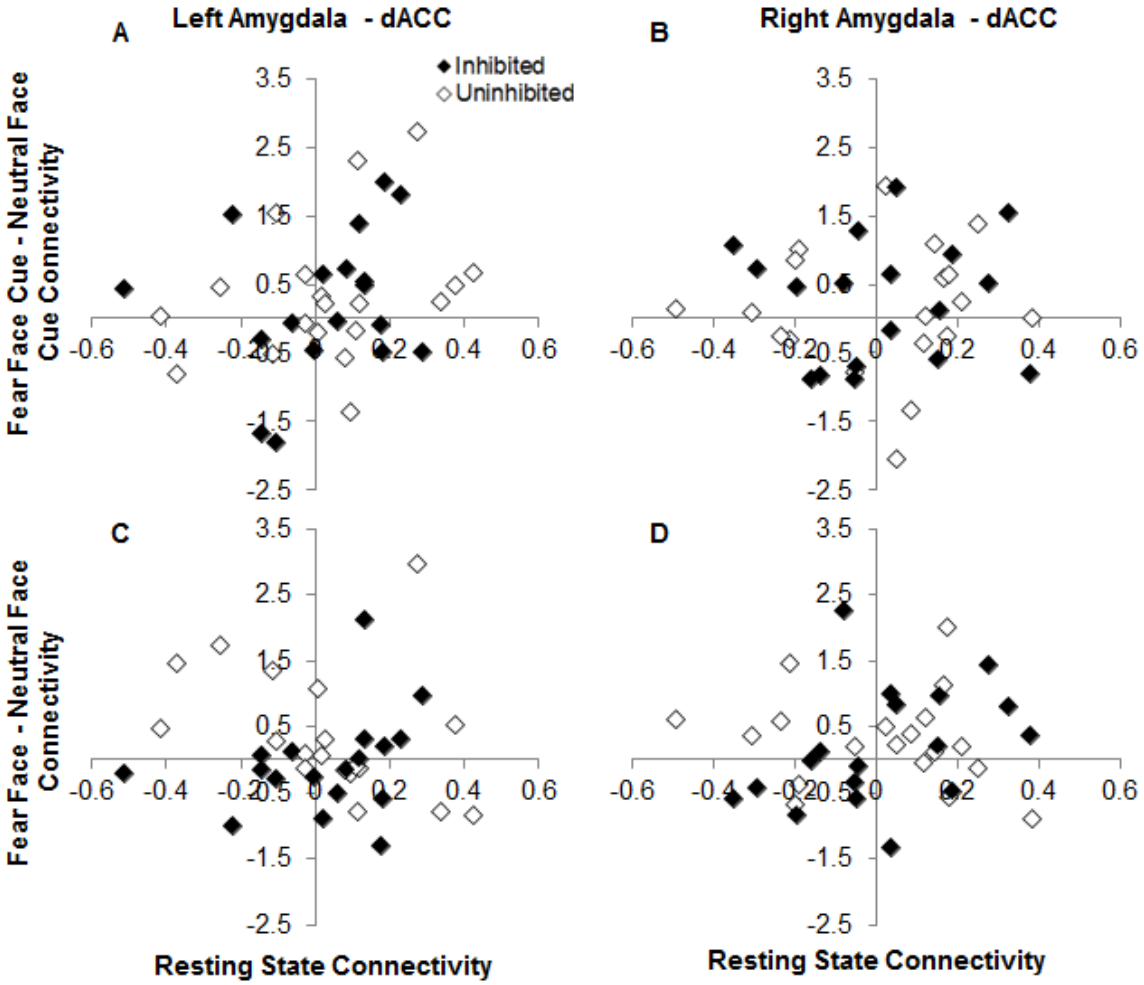
	IT		UT		
	Mean	SD	Mean	SD	p-value
Task-Based Connectivity Fear Face Cue – Neutral Face Cue					
Left Amygdala	.23	1.06	.33	.99	.76
Right Amygdala	.27	.90	.14	.94	.13
Task-Based Connectivity Fear Face – Neutral Face					
Left Amygdala	-.07	.77	.38	.97	.67
Right Amygdala	.18	.90	.30	.72	.64
Resting State Connectivity					
Left Amygdala	.03	.26	.11	.27	.33
Right Amygdala	-.06	.24	.01	.23	.37

Note: IT = inhibited temperament; UT = uninhibited temperament; SD = standard deviation

connectivity, we tested for a correlation between the two measures. There were no significant correlations between amygdala-dACC resting state connectivity and connectivity during anticipation of fear faces, relative to

anticipation of neutral faces (left amygdala: r = .26, p = .13, Figure 13A; right amygdala: r = .04, p = .83, Figure 13B) or during face viewing (left amygdala: r = -.09, p = .61, Figure 13C; right amygdala: r = .12, p = .49, Figure 13D). Finally, to determine if there was a relationship between task-based and resting state connectivity within either group specifically, we tested for a correlation within both the inhibited group and the uninhibited group. Within the inhibited temperament group, there were no significant correlations between amygdala-dACC resting state connectivity and connectivity during anticipation of fear faces, relative to anticipation of neutral faces (left amygdala: r = .21, p = .41; right amygdala: r = .02, p = .94) or during fear face viewing, relative to neutral face viewing (left amygdala: r = .28, p = .26; right amygdala: r = .39, p = .10). Finally, within the uninhibited temperament group, there were no significant correlations between amygdala-dACC resting state connectivity and connectivity during anticipation of fear faces, relative to anticipation of neutral faces (left amygdala: r = .30, p = .21; right amygdala: r = .05, p = .83) or during fear face viewing, relative to neutral face viewing (left amygdala: r = -.34, p = .16; right amygdala: r = -.17, p = .48).

Figure 13. Correlation of amygdala-dACC connectivity during task with resting state.



There was no correlation between: **A)** left amygdala-dACC connectivity during anticipation of fear faces, relative to anticipation of neutral faces, and left amygdala-dACC resting state connectivity ($r = .26$, $p = .13$); **B)** right amygdala-dACC connectivity during anticipation of fear faces, relative to anticipation of neutral faces, and right amygdala-dACC resting state connectivity ($r = .04$, $p = .83$); **C)** left amygdala-dACC connectivity during fear face viewing, relative to neutral face viewing, and left amygdala-dACC resting state connectivity ($r = -.09$, $p = .61$); **D)** right amygdala-dACC connectivity during fear face viewing, relative to neutral face viewing, and right amygdala-dACC resting state connectivity ($r = .12$, $p = .49$).

Discussion

We hypothesized that inhibited children would have less amygdala regulation by the prefrontal cortex, as measured by less amygdala-dACC

connectivity during the anticipation of fear faces, viewing of fear faces, and at rest. We proposed that amygdala-dACC connectivity might differ between temperament groups based on two findings in inhibited adults: 1) inhibited adults had less connectivity between the amygdala and dACC at rest (Blackford et al., 2014); 2) inhibited adults had less dACC activation when viewing expected blocks of fear faces (Clauss et al., 2011). However, both groups had similar patterns of amygdala-dACC connectivity. We also hypothesized that alterations in amygdala-dACC connectivity might be based in alterations in resting state connectivity, but there was no relationship between the resting state and task-based connectivity.

During anticipation of fear faces and viewing of fear faces, there were no significant differences in amygdala-dACC connectivity. There are several possibilities for why we had a negative finding. First, we may have found differences in connectivity using different amygdala and dACC seed regions. Seed regions can be defined using an anatomical mask, such as a whole amygdala mask or an amygdala subnucleus mask. The amygdala is a heterogeneous structure composed of multiple subnuclei, each subnucleus has a unique pattern of connectivity (Aggleton et al., 1980; deCampo and Fudge, 2013; Price and Amaral, 1981; Roy et al., 2009). Children with anxiety symptoms and adults with an inhibited temperament have alterations in amygdala connectivity that differ by subnucleus (Blackford et al., 2014; Etkin et al., 2009; Qin et al., 2014; Roy et al., 2013). Additionally, the basolateral amygdala has stronger structural connectivity with the ACC (Ghashghaei et al., 2007; Morecraft et al.,

2007); we may have found significant connectivity and temperament differences if we had tested for differences in basolateral amygdala-dACC connectivity. Seed regions can also be defined using a mask from functional differences, such as a sphere around the peak temperament difference in activation. We may have found differences in amygdala-dACC connectivity if we defined our mask based on other functional criteria. Finally, our original hypothesis was based on data from adults with an inhibited temperament.

DACC functional connectivity undergoes developmental changes, shifting from diffuse connections, to more focal connections (Kelly et al., 2008), we may only identify differences in amygdala-dACC connectivity in adults; however, developmental differences in amygdala-dACC connectivity specifically have yet to be examined. Several studies have shown developmental changes in connectivity between the amygdala and several other PFC regions. When viewing fear faces, connectivity between the amygdala and the rostral ACC is positive in young children and becomes negative around age 10 (Gee et al., 2013b). At rest, children have less connectivity between the amygdala and the dlPFC and rACC than adults (Qin et al., 2012). Developmental changes in connectivity are also impacted by environment; for example, children who have lived in institutional care have negative amygdala-rACC throughout childhood and adolescence (Gee et al., 2013a). Recent evidence also shows that mice that react more strongly to social stressors also have alterations in amygdala-infralimbic cortex (rodent analogue of ACC) connectivity (Kumar et al., 2014). These findings suggest that in children at risk for developing psychopathology

broadly, the trajectory of amygdala-prefrontal cortex development is altered. This study only examined at-risk children at one time point; future studies are needed to determine whether inhibited children show different developmental trajectories of amygdala-dACC connectivity.

Several previous studies have found a relationship between task-based and resting state connectivity; however, in this study, we found no relationship between amygdala-dACC connectivity during task-based and resting state connectivity. Activation during a simple motor task correlates with resting state connectivity (Fox et al., 2007, 2006). Patients with social anxiety disorder have less amygdala-rostral ACC connectivity during both a face processing task and at rest, and with significant overlap between these findings (Prater et al., 2013). These prior studies have all compared task-based and resting state connectivity in adults, rather than children. Adults have stronger amygdala functional connectivity and have more segregation of amygdala networks (Qin et al., 2012). As functional networks are still developing in children, we may not find a relationship between task-based and resting state connectivity.

Conclusions

We found no significant differences in amygdala-dACC connectivity between groups, and no relationship between task-based and resting state connectivity. Given that the prefrontal cortex undergoes a protracted development over childhood and adolescence (Casey et al., 2000; Gogtay et al., 2004) and that amygdala-prefrontal cortex connectivity also undergoes

developmental changes (Gee et al., 2013b; Kelly et al., 2008; Qin et al., 2012), amygdala-dACC connectivity may not yet be fully established in the 8-10 year old children in this study. Future studies should examine the development of amygdala-dACC connectivity to determine when functional and structural connections are formed. Children with anxiety have alterations in amygdala subnuclei networks (Qin et al., 2014; Roy et al., 2013); future studies in inhibited temperament should examine differences in both amygdala subnuclei connectivity and in connectivity with different regions of the ACC. Finally, trajectories of prefrontal cortex development in inhibited and uninhibited children likely differ, however, to date, no studies have examined that question. Future studies should test for differences in prefrontal cortex development in inhibited children to understand how social anxiety develops in inhibited children.

CHAPTER V

ALTERATIONS IN BED NUCLEUS OF THE STRIA TERMINALIS FUNCTIONAL CONNECTIVITY

Introduction

The bed nucleus of the stria terminalis (BNST) is a small subcortical structure that is part of the "extended amygdala" (Olmos and Heimer, 1999). The BNST is primarily known for its function in responding to uncertain or sustained threat (Davis, 1998; Davis et al., 1997). Work by Davis and others (Davis, 1998; Davis et al., 2009) has conclusively shown that the BNST mediates response to a sustained threat, such as exposing a rodent to a bright light or putting it in a context where it has previously received a shock; in contrast, the amygdala mediates response to a conditioned fear stimulus, such as exposing a rodent to a light that was previously paired with a shock. Additionally, the amygdala and BNST respond differently to pharmacological treatments (Miles et al., 2011), suggesting that modifying BNST response may be an additional target for treatment of anxiety disorders. In the study of inhibited temperament and anxiety disorders, the amygdala has been the primary region of focus, but there is increasing evidence to suggest a role for the BNST in inhibited temperament and anxiety. However emerging evidence from non-human primates shows that when non-human primates are exposed to a novel social situation, more inhibited primates have increased BNST activation (Fox et al., 2010, 2008).

Recently, neuroimaging studies in humans and non-human primates have confirmed decades of findings in rodents showing that the BNST is activated in response to sustained threat or anxiety. The BNST is activated when healthy adults view unpredictable, aversive images (i.e. sharks, dental work) or when adults anticipate an unpredictable shock (Alvarez et al., 2011; Somerville et al., 2012). Additionally, adults with more anxiety have increased BNST activity when anticipating a painful shock (Somerville et al., 2010). When patients with spider phobia anticipate seeing a spider that could appear at any time, they have increased BNST activation (Straube et al., 2007). Given that much of the behavior in inhibited temperament is based on a response to a novel or uncertain stimulus, the elevated BNST activation is likely to contribute to inhibited temperament; however, differences in BNST activation and neurocircuitry have yet to be examined in humans with an inhibited temperament.

The BNST has connectivity with a number of brain regions implicated in anxiety, including the amygdala (Avery et al., 2014; deCampo and Fudge, 2013; Oler et al., 2012), and regions that mediate the peripheral effects of anxiety, such as the hypothalamus, brainstem, and insula (Dong and Swanson, 2006, 2004; Dong et al., 2001, 2000). Recently, our lab has developed techniques to test for differences in BNST functional connectivity in humans (Avery et al., 2014). Testing for differences in BNST functional connectivity—may provide new insight into inhibited temperament and risk for social anxiety disorder.

In this study, we tested for temperament differences in BNST connectivity across the brain using functional connectivity measures during an emotional task

(anticipation and viewing of faces) and a non-emotional task (resting state). We hypothesized that inhibited children would show increased BNST connectivity across the brain.

Methods

Recruitment

Study participants were 40 children (20 inhibited, 20 uninhibited) who were recruited for: 1) being between the ages of 8-10 years; 2) having no contraindications for MRI; and 3) having no history of psychiatric diagnosis or treatment (see Chapter 2 for details on recruitment and screening). There were no differences between temperament groups in age, gender, ethnicity, or handedness (see Table 3).

Experimental Design

Data were collected and processed as detailed in Chapter 4. Two types of functional connectivity data were collected. Task-based functional connectivity data were collected from a social anticipation task and processed as detailed in Chapter 4. Resting state connectivity data were collected and preprocessed as detailed in Chapter 4.

Task-Based Functional Connectivity Data

To identify temperament differences in BNST task-based functional

connectivity, we used psychophysiological interaction analysis (PPI; Friston et al., 1997). The BNST seed region was from a previously published mask (Avery et al., 2014). Using the generalized psychophysiological toolbox (gPPI; http://www.brainmap.wisc.edu/PPI), average BOLD time series were extracted from the right and left BNST seed regions. PPI analysis uses three regressors: the physiological regressor, the psychological regressor, and the interaction regressor. The physiological regressor was the BOLD time series, and the psychological regressor was one of the following contrasts: fear face anticipation – neutral face anticipation, fear face anticipation – neutral object anticipation, neutral face anticipation – neutral object anticipation, fear face viewing – neutral face viewing, fear face viewing – neutral object viewing, or neutral face viewing – neutral object viewing. The interaction regressor modeled the change in BNST connectivity between the conditions in the contrast. The regressors were used to model the BOLD time series in individual subjects, producing an estimate of differences in connectivity at each voxel. The resulting contrast images were used to test for differences in connectivity.

Resting State Functional Connectivity Data

Resting state functional connectivity data were acquired and processed as detailed in Chapter 4. Intrinsic resting state connectivity was calculated using the CONN toolbox (Whitfield-Gabrieli and Nieto-Castanon, 2012). The BNST seed region was from a previously published mask (Avery et al., 2014).

Data Analysis

To test for temperament differences in BNST connectivity across the brain, independent-samples t-tests were performed in SPM8 to determine whether connectivity differed as a function of temperament. Cluster-based thresholding was used to adjust for multiple comparisons. Based on simulations performed with AlphaSim (http://afni.nimh.nih.gov/pub/dist/doc/manual/AlphaSim.pdf), a family-wise error rate of $\alpha \leq 0.05$ was achieved with a voxel threshold of $p < .005$ and $k > 90$. For post hoc analysis, beta values from regions with significant temperament differences in connectivity were extracted using the Easy ROI tool.

Results

Task-Based Functional Connectivity

During anticipation of fear faces, relative to anticipation of neutral faces, the inhibited children had significantly greater BNST connectivity with a region of the parietal cortex ($p < .05$, corrected, see Figure 14 and Table 10). During anticipation of fear faces, relative to anticipation of neutral objects, inhibited children had significantly greater BNST connectivity with the visual cortex, parietal cortex, and cerebellum (all $p < .05$, corrected). During anticipation of neutral faces, relative to anticipation of neutral objects, inhibited children had greater BNST connectivity with the dorsomedial prefrontal cortex ($p < .05$, corrected). There were no significant temperament differences in BNST

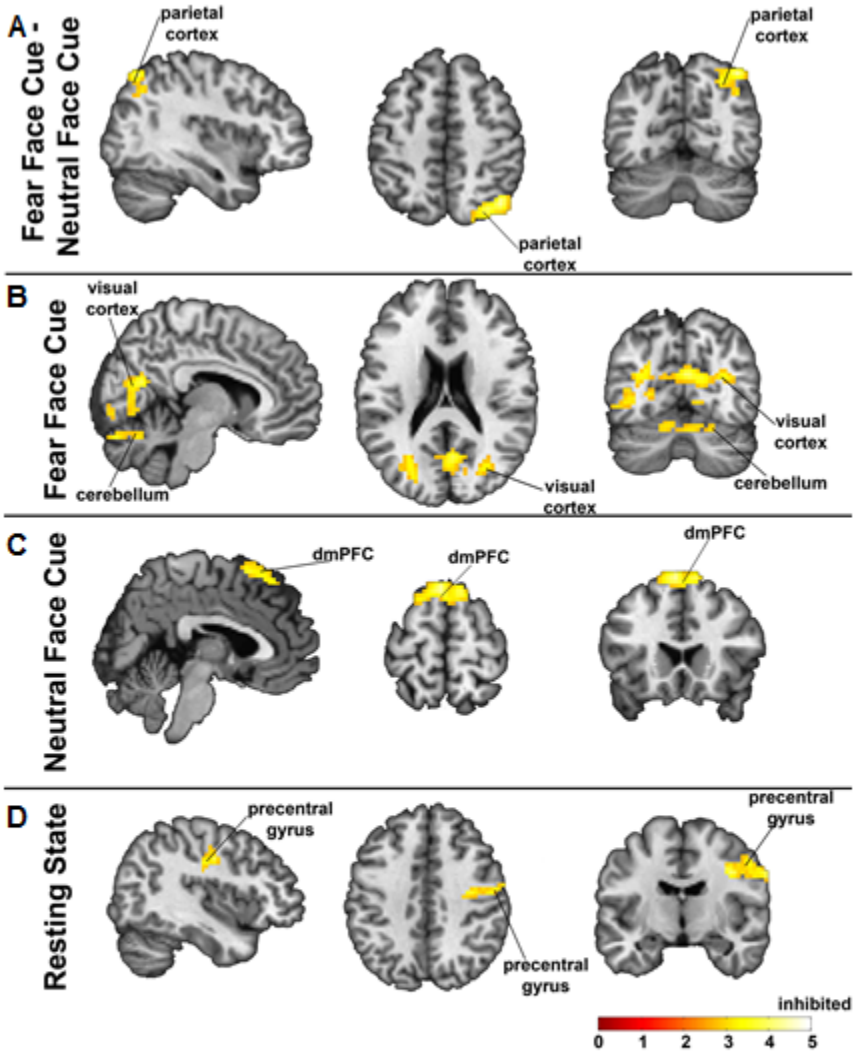
connectivity during face viewing. There were no regions that had significantly greater BNST connectivity in the uninhibited children.

Table 10. Temperament differences in BNST connectivity.

BNST Hemisphere	Target Region (Hemisphere)	Brodmann Area	Cluster Size (voxels)	Z Score	Peak Voxel x	y	z
Fear Face Cue > Neutral Face Cue							
Inhibited Temperament > Uninhibited Temperament							
Left	Parietal cortex (R)	7, 39	143	3.85	42	-67	52
Fear Face Cue > Neutral Object Cue							
Inhibited Temperament > Uninhibited Temperament							
Right	Middle occipital gyrus, middle temporal gyrus, superior temporal gyrus (L)	18, 19, 30	450	3.74	-36	-43	16
Right	Calcarine fissure, precuneus, cuneus, middle occipital gyrus (B)	18, 31	495	3.70	6	-64	19
Right	Cerebellum (R)		194	3.36	21	-73	-26
Neutral Face Cue > Neutral Object Cue							
Inhibited Temperament > Uninhibited Temperament							
Right	Dorsomedial prefrontal cortex (B)	6, 8	173	3.82	-3	20	67
Resting State Connectivity							
Inhibited Temperament > Uninhibited Temperament							
Right	Precentral gyrus, postcentral gyrus (R)	4, 6	142	3.62	39	-9	36

Note: IT = inhibited temperament; UT = uninhibited temperament; SD = standard deviation

Figure 14. Temperament differences in BNST connectivity.



A) During anticipation of fear faces, relative to anticipation of neutral faces, inhibited children had greater BNST connectivity with the parietal cortex. **B)** During anticipation of fear faces, relative to anticipation of neutral objects, inhibited children had greater BNST connectivity with the visual cortex and cerebellum. **C)** During anticipation of neutral faces, relative to neutral objects, inhibited children had greater BNST connectivity with the dorsomedial prefrontal cortex. **D)** At rest, inhibited children had greater BNST connectivity with the precentral gyrus or primary motor cortex.

Resting State Functional Connectivity

At rest, the inhibited children had significantly greater BNST connectivity with the motor cortex, including the pre-central and post-central gyri ($p < .05$, corrected; see Figure 16D and Table 10).

Discussion

To date, no studies have examined BNST neurocircuitry in children or in inhibited temperament in humans; however, non-human primates with inhibited temperament have increased activation of the bed nucleus of the stria terminalis (BNST) and studies in rodents have shown that the BNST is critical for production of anxiety behavior. We tested for temperament differences in BNST neurocircuitry across the brain during a task, the anticipation and viewing of fear faces and neutral faces. We also tested for temperament differences in BNST neurocircuitry at rest. We found that during anticipation of viewing fear faces, inhibited children had increased BNST connectivity with regions involved in visual processing—visual and parietal cortex. During anticipation of neutral faces, which may be more salient to inhibited children, inhibited children had increased BNST connectivity with a region implicated in emotion regulation—the dorsomedial prefrontal cortex. At rest, inhibited children had greater BNST connectivity with the primary motor cortex—this increased connectivity may result in increased freezing behavior in children with an inhibited temperament (Garcia-Coll et al., 1984). This study is the first evidence that BNST neurocircuitry is altered in children with an inhibited temperament, who are at high risk for developing social anxiety disorders.

During anticipation of viewing fear faces, inhibited children had stronger BNST connectivity with both primary visual cortex and with secondary visual processing areas, including posterior parietal cortex. We propose that this

increased connectivity likely occurs through a BNST-amygdala-visual cortex-parietal cortex neural circuit. While there are no known structural connections between the BNST and the visual cortex, the amygdala has significant connectivity with both the BNST (Avery et al., 2014; deCampo and Fudge, 2013; Dong et al., 2001; Oler et al., 2012) and the visual cortex (Amaral et al., 2003). The amygdala receives visual input through dense connections from the visual cortex and also activates visual cortex to signal attention to salient events (Phelps and LeDoux, 2005). The visual cortex projects to the posterior parietal cortex for further visual processing (Baizer et al., 1991). Additionally, adults with an inhibited temperament have increased resting state connectivity between the central nucleus of the amygdala and a similar region of the parietal cortex to the region we found in this study (Blackford et al., 2014), providing further evidence for upregulation of a BNST-amygdala-visual cortex-parietal cortex circuit.

Upregulation of BNST-visual processing pathways during anticipation of viewing faces likely represents increased visual attention and preparation for viewing salient stimuli—fear faces—in inhibited children. In rodents and humans, the BNST is activated during periods of sustained anxiety or uncertain threat and rodents, the BNST is critical for the production of light-enhanced startle (Davis, 1998), reinforcing the idea that BNST-visual pathways are important in sustained anxiety responses. This increased BNST-visual connectivity during anticipation may cause greater visual cortex activation found in inhibited children during fear face viewing (see Chapter 3). Inhibited adults also have increased visual cortex activation while anticipating faces (Clauss et al., 2014a) and viewing faces

(Blackford et al., 2011; Schwartz et al., 2003a). Previous findings of increased visual cortex activity may be mediated by increased amygdala-visual cortex or BNST-visual cortex connectivity; however differences in connectivity were not tested in those paradigms.

During anticipation of viewing neutral faces, inhibited children had increased connectivity between the BNST and dorsomedial prefrontal cortex (dmPFC). The dmPFC has functions in emotion regulation (Diekhof et al., 2011; Hermann et al., 2009; Kober et al., 2008; Ochsner et al., 2004). No studies in primates or rodents have found structural connections between the BNST and the dmPFC; however, our lab found significant functional connectivity between the BNST and the dmPFC (Avery et al., 2014). Neutral faces are particularly salient to inhibited temperament. We propose that increased BNST-dmPFC connectivity in inhibited children during anticipation of neutral faces may be a mechanism by which the BNST primes the prefrontal cortex to respond to upcoming neutral faces stimuli in the inhibited temperament group.

At rest, inhibited children had greater connectivity between the BNST and the primary motor cortex, which may be related to increased freezing behavior in inhibited temperament. Children with an inhibited temperament are more likely to freeze when they encounter novel or unfamiliar situations (Garcia-Coll et al., 1984; Kagan and Snidman, 2004) and non-human primates and rodents with an inhibited temperament display increased freezing duration (Jiao et al., 2011; Kalin and Shelton, 2003; Stead et al., 2006). In non-human primates, the duration of freezing is positively correlated with activation in a region of the

primary motor cortex (Shackman et al., 2013) that is very similar to the region we identified as having stronger BNST connectivity in inhibited temperament. The BNST projects to a number of regions that control motor output, including the nucleus accumbens and thalamic nuclei (Dong and Swanson, 2004), as well as to the periaqueductal gray (Dong et al., 2000). While the BNST and the motor cortex are not known to be structurally connected, the BNST projects to the nucleus accumbens, which then projects to the globus pallidus (Nauta et al., 1978); the globus pallidus inhibits motor cortex via the ventral thalamus (DeVito and Anderson, 1982). Increased BNST functional connectivity with motor cortex may be through a BNST-nucleus accumbens-globus pallidus-thalamus-motor cortex pathway; this increased BNST connectivity with motor cortex at rest may mediate increased freezing in inhibited children.

Little is known about the connectivity of the BNST in primates. The majority of the work on BNST connectivity has been in rodent models and has shown that the BNST is structurally connected with limbic regions; few studies have shown that the BNST is connected with cortical regions, but it is known to be connected with infralimbic cortex (ACC in humans) (Dong and Swanson, 2006, 2004; Dong et al., 2001, 2000). Recently, our lab published the first study of BNST connectivity in healthy adults; however, no studies have tested for differences in BNST connectivity over development or in inhibited adults.

Conclusions

During anticipation of viewing faces, children with an inhibited temperament had increased BNST connectivity to regions involved in the processing of visual stimuli and cognitive control. When inhibited children viewed faces, they had increased activation of the visual cortex and dmPFC, suggesting that increased functional connectivity to the BNST may represent increased signaling between the BNST and those regions. The BNST mediates sustained anxiety and uncertain threat, thus BNST functional connectivity with these regions may upregulate activation during the viewing of faces. Finally, inhibited children had increased BNST connectivity with the motor cortex at rest, which may be the mechanism by which inhibited temperament is associated with increased freezing behavior. It remains unknown if BNST connectivity is malleable and can be changed by experience or medication. Future studies should examine changes in BNST connectivity over development and in patients who have developed social anxiety disorder to understand how BNST connectivity may contribute to the development of social anxiety disorder.

CHAPTER VI

SYNOPSIS AND CONCLUSIONS

Synopsis

Inhibited temperament is a biologically-based risk factor for the development of anxiety disorders (Biederman et al., 2001, 1993, 1990; Chronis-Tuscano et al., 2009; Clauss and Blackford, 2012; Essex et al., 2010; Hirshfeld et al., 1992; Rosenbaum et al., 1991; Schwartz et al., 1999). In adolescents and young adults, inhibited temperament is associated with amygdala hyperactivity and alterations in prefrontal cortex activation (Blackford et al., 2013, 2011, 2009; Clauss et al., 2014a, 2014b; Pérez-Edgar et al., 2007; Schwartz et al., 2012, 2003a). Recent studies have shown that inhibited adults with more anxiety symptoms have less prefrontal cortex activation during cognitive control processes; decresed prefrontal cortex activation may be a neural vulnerability factor for anxiety disorders in inhibited adults (Clauss et al., 2014a; Jarcho et al., 2013a).

In this study, we found that inhibited children who had not yet developed anxiety disorders had underlying neural vulnerability factors, including increased amygdala activation and decreased prefrontal cortex activation during anticipation of faces. In contrast to the anticipation period, during the face viewing period, inhibited children had increased prefrontal cortex activity. These prefrontal cortex findings suggest that inhibited children may have a delay in

activation of emotion regulation or cognitive control, or that they fail to effectively prepare to see social stimuli.

One part of the prefrontal cortex in particular—the dorsal anterior cingulate cortex (dACC)—has been consistently implicated in inhibited temperament in adults and has functions in cognitive control (Clauss et al., 2014a, 2011; Etkin and Wager, 2007). Adults with an inhibited temperament have less functional connectivity between the dACC and the amygdala (Blackford et al., 2014). Unlike adults, inhibited and uninhibited children had similar patterns of connectivity between the dorsal anterior cingulate cortex (dACC) and the amygdala. These findings suggest that while differences in activation of cognitive control regions are present in children with an inhibited temperament, these findings are not related to differences in amygdala-dACC connectivity. Differences in connectivity between the amygdala and other parts of the anterior cingulate cortex or prefrontal cortex may be present and should be explored in future studies.

Finally, we tested for differences in connectivity with the bed nucleus of the stria terminalis (BNST), a small subcortical region that mediates sustained responses to threat and anxiety. BNST connectivity differences were tested during task and at rest. Inhibited children had increased BNST connectivity with regions involved in visual processing during anticipation of viewing fear faces, and increased BNST connectivity with regions involved in cognitive control and emotion regulation during anticipation of viewing neutral faces. Inhibited children and adults have greater visual cortex activity when viewing faces (see Chapter 3; Blackford et al., 2011; Schwartz et al., 2003a), and this increased activation may

be through BNST-amygdala-visual cortex pathways. At rest, the inhibited children had increased BNST connectivity with the motor cortex, which may be the mechanism by which inhibited temperament is associated with increased freezing behavior.

Inhibited children show alterations in a limbic-frontal circuit, including the amygdala, insula, rostral anterior cingulate cortex, dorsolateral prefrontal cortex, and dorsomedial prefrontal cortex. These alterations include increased activity of the amygdala and insula and differences in timing of prefrontal cortex activation. This neural signature of inhibited temperament is independent of anxiety disorders, as we found these differences in 8-10 years old children who have yet to develop anxiety disorders.

Conclusions

We have demonstrated broad differences in amygdala-prefrontal cortex neurocircuitry in children with an inhibited temperament. These findings are in the context of broad changes in neurocircuitry as the brain matures. The prefrontal cortex undergoes many changes throughout development—including proliferation, synaptic pruning, and myelination (Gogtay et al., 2004; Huttenlocher, 2002)—and these changes correspond to increases in cognitive control and emotion regulation abilities (Casey et al., 2000). When young, healthy control children view fear faces, amygdala-anterior cingulate cortex connectivity is positive and shifts to an adult pattern of negative connectivity around the age of 10 (Gee et al., 2013b). Concurrent with this shift in connectivity

is a reduction in amygdala activation (Gee et al., 2013b). In inhibited children who are vulnerable to anxiety disorders, amygdala-anterior cingulate connectivity may not shift. In inhibited children who are resilient to anxiety disorders, amygdala-anterior cingulate connectivity may follow the typical pattern and become negative; however, these hypotheses have yet to be tested. Children with an inhibited temperament had less prefrontal cortex activation during anticipation of viewing fear faces; however adults with an inhibited temperament had greater prefrontal cortex activation during anticipation of viewing fear faces (Clauss et al., 2014a). For inhibited adults, increased anterior cingulate activity is beneficial and corresponds to fewer social anxiety symptoms (Clauss et al., 2014a). In inhibited children, failure to engage prefrontal cortex during anticipation of negative social stimuli, such as fear faces, is an underlying vulnerability factor and may be a neural signature of risk for social anxiety disorder.

We hypothesize that as the prefrontal cortex continues to mature, some inhibited children will begin to engage prefrontal anticipatory processes, and will be resilient to developing anxiety disorders. We predict that inhibited children who do not develop the ability to engage prefrontal cortex during anticipation will be vulnerable to developing anxiety disorders. What might cause some inhibited children to develop increased anticipatory prefrontal cortex activation as they mature? Differences in prefrontal cortex maturation are likely a result of both nature—including underlying genetic differences in prefrontal cortex development—and nurture—including exposure to positive environments, such

as moderate challenges or beneficial school environments, or exposure to negative environments, including stress and trauma. Children with an inhibited temperament have fewer anxiety symptoms if their parents are warm, but firm (Williams et al., 2009); however, it remains unknown by what mechanism parenting prevents the development of anxiety symptoms. In rodent models, both enriched environments and attentive maternal care can induce neural proliferation and synaptogenesis (Kaffman and Meaney, 2007; Nithianantharajah and Hannan, 2006); targeted interventions that address both parenting and the child's environment may induce prefrontal cortex proliferation in inhibited children. Longitudinal studies that follow inhibited children over time are critical for dissecting the complex brain and environment factors that lead to anxiety disorders.

Anticipatory prefrontal cortex activation may be modified by treatments and could be a target for intervention in high-risk inhibited children. Activity of the prefrontal cortex can be modified by psychotherapy (Klumpp et al., 2013a), and drug treatments (Aupperle et al., 2011). Given that the prefrontal cortex undergoes protracted development over time (Casey et al., 2000; Huttenlocher, 2002), there may be particular critical periods during which interventions are most effective. To date, no studies have examined interventions developmentally, but this is a critical future direction for psychiatric research. If critical periods for prefrontal cortex development could be identified, targeted interventions could be developed for inhibited children.

We hypothesized that inhibited children would show heightened amygdala activation to fear faces, consistent with previous studies of inhibited adolescents and adults. However, in this study, inhibited children had less discrimination in amygdala activation—inhibited children had similar patterns of amygdala activation during anticipation of viewing both fear and neutral faces. Increased amygdala response has been consistently identified in inhibited adolescents and adults (Blackford et al., 2013, 2011, 2009; Clauss et al., 2014b, 2011; Pérez-Edgar et al., 2007; Schwartz et al., 2012, 2003a). Inhibited adults also fail to discriminate between novel and familiar faces (Blackford et al., 2011), suggesting that inhibited temperament may be associated with amygdala generalization to social stimuli. Increased generalization has been proposed as an underlying mechanism of anxiety disorders (Lissek et al., 2010) and inhibited children may have an underlying hypersensitivity to faces, which leads to increased amygdala activation to faces in general. In inhibited temperament, faces are likely to be more salient or threatening, which leads to increased amygdala activation to all faces, and may lead to the development of social anxiety disorder.

Another explanation for the lack of amygdala differences in inhibited children is that the amygdala undergoes structural and functional changes across development and inhibited children may have alternate developmental trajectories. The amygdala develops early in life, during the fetal and early postnatal period (Chareyron et al., 2012). In non-human primate models, lesioning the amygdala during the postnatal period increases fear behavior (Raper et al., 2013), but lesioning the amygdala during adulthood decreases fear

behavior (Fox et al., 2012; Kalin et al., 2001; Machado et al., 2009). The functional activation of the amygdala also changes over childhood. Amygdala activation to faces decreases as children grow older (Gee et al., 2013b; Killgore et al., 2001). However, amygdala activation in inhibited adults remains high (Blackford et al., 2013, 2011, 2009; Pérez-Edgar et al., 2007; Schwartz et al., 2003a), suggesting that at some point in development, the trajectories of inhibited children may diverge from the typical pattern. Inhibited adults have increased amygdala volume, which correlates with increased amygdala activity (Clauss et al., 2014b). Evidence from rodent models points to a role for chronic stress in increased amygdala volume (Mitra et al., 2005; Vyas et al., 2002), and inhibited individuals may have increased amygdala volume due to chronic anxiety and stress. Increased amygdala volume may cause increased amygdala activation and hyperactivity. Inhibited children and adults have increased amygdala response, which may stem from amygdala hypertrophy; this amygdala hypertrophy is due to exposure to chronic anxiety and stress.

Within the context of developmental neurobiology, our findings fit with both the Casey model of fear neurocircuitry development (Casey et al., 2013) and the Ernst triadic model of motivated behavior (Ernst and Fudge, 2009; Ernst et al., 2005). Both models suggest that a relative imbalance in the development of prefrontal cortical and subcortical regions contributes to the behavioral changes observed during through childhood and adolescence. In children and adolescents, subcortical brain regions, such as the amygdala, mature earlier, whereas the prefrontal cortex matures later. This differential maturation leads to

a relative imbalance between the amygdala, which responds to fear stimuli, and the prefrontal cortex, which regulates amygdala response. During adolescence, this differential maturity between the amygdala and prefrontal cortex is accompanied by a corresponding increase in the prevalence of anxiety disorders (Kessler et al., 2005). In the current study, we found evidence of an amygdala-prefrontal cortex imbalance during anticipation; inhibited children had a similarly increased amygdala response during anticipation of both face types, accompanied by less prefrontal cortex regulation. These models predict that for most children, prefrontal cortex development will eventually catch up with the amygdala and restore balance between the two regions, but for some children, the amygdala and prefrontal cortex imbalance will persist. This imbalance will lead to the development of anxiety disorders.

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