INDIVIDUAL DIFFERENCES IN ASSOCIATIVE LEARNING, INTRINSIC CONNECTIVITY AND NEURAL REACTIVITY: SUPPORT FOR A CEREBELLAR ROLE IN ANXIETY VULNERABILITY

Meghan Davis Caulfield, M.S.

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Table of Contents

Table of Contents ............................................................... 2
Table of Tables ..................................................................... 4
Table of Figures ..................................................................... 5
Acknowledgements .............................................................. 6
Abstract .................................................................................. 7

A. Introduction
   I. A diathesis-stress approach: Role of behavioral inhibition ....... 8
   II. The cerebellum: Structure and function ................................ 10
   III. Classical eyeblink conditioning ........................................... 11
   IV. Higher cognitive processes of the cerebellum ...................... 14
   V. Neural substrates underlying anxiety vulnerability .............. 17
   VI. Rationale .......................................................................... 18
   VII. Outline of research studies .............................................. 19

B. Experimental Results
   I. Classical Conditioning
      Study 1. Facilitated acquisition of eyeblink conditioning in those vulnerable to anxiety disorders ......................................................... 21
      Study 2. Facilitated acquisition of standard but not long delay classical eyeblink conditioning in behaviorally inhibited adolescents .......... 47
II. Functional Magnetic Resonance Imaging

Study 3. Individual differences in resting-state functional connectivity with the executive network: Support for a cerebellar role in anxiety vulnerability……………………………………………………………………64

Study 4. Differential activation to familiar and novel stimuli in those vulnerable to anxiety disorders…………………………………………………84

C. Conclusion

I. Classical conditioning…………………………………………………………104

II. Functional neuroimaging……………………………………………………107

III. Summary……………………………………………………………………112

D. References……………………………………………………………………114
Table of Tables

1.1 Descriptive summary of scores on self-report scales………………………………………42

1.2 Relationship of self-report measures of anxiety vulnerability (n=174)…………………43

1.3 Summary of eyeblink conditioning groups made for comparison of high and low scores on AMBI, RMBI, CSRI, RSRI, STAI-Trait and STAI-State (n=117)………………………………………………………………………………………………………………………………………………………………………………………………………………44

2.1 Number of participants and mean score on the Adult Measure of Behavioural Inhibition in each condition………………………………………………………………………………………………………………………………………………………………………………………………………………60

3.1 Group mean demographic details………………………………………………………………………………78

3.2 Significantly connected regions with the dorsolateral prefrontal cortex……………79

3.3 Regions of greater connectivity for high AMBI compared to low AMBI……………80

4.1 Group mean demographic details………………………………………………………………………………98

4.2 Mean reaction times and corrected recognition between groups…………………99

4.3 Differentially active regions from Whole-brain Group Analysis…………………100
Table of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.1</td>
<td>The intrinsic cerebellar circuitry is necessary and sufficient for acquisition of delay classical eyeblink conditioning</td>
</tr>
<tr>
<td>1.1</td>
<td>Eyeblink conditioned responding of high and low scoring groups</td>
</tr>
<tr>
<td>1.2</td>
<td>Eyeblink conditioned responding of the upper and lower 1/3 of scores on AMBI and Trait</td>
</tr>
<tr>
<td>2.1</td>
<td>Acquisition of the eyeblink CR for groups receiving a 500-ms or 1000-ms paired CS duration</td>
</tr>
<tr>
<td>2.2</td>
<td>Acquisition of the eyeblink CR for 500-ms (left panel) and 1000-ms paired (right panel) as a function of AMBI group</td>
</tr>
<tr>
<td>2.3</td>
<td>Acquisition of the eyeblink CR for 1000-ms explicitly unpaired compared to paired eyeblink conditioning</td>
</tr>
<tr>
<td>3.1</td>
<td>Matching slices (x= -24, y= -68, z= -69) demonstrate similar connectivity between the cerebellum and executive network seeds</td>
</tr>
<tr>
<td>3.2</td>
<td>The cerebellum Crus I (x= -40, y=-49 z= -41 shown here) also showed significant correlations of intrinsic activity with the orbitofrontal insula seed of the salience network</td>
</tr>
<tr>
<td>3.3</td>
<td>Between-group comparisons indicated significantly greater cerebellar connectivity of the Crus I (34, -73, -36) with the executive network in behaviorally inhibited individuals</td>
</tr>
<tr>
<td>4.1</td>
<td>Samples of face and scene stimuli</td>
</tr>
<tr>
<td>4.2</td>
<td>Recognition accuracy of stimuli by group</td>
</tr>
<tr>
<td>4.3</td>
<td>Brain activity for high AMBI and low AMBI groups</td>
</tr>
<tr>
<td>C.I.</td>
<td>First ten trials of paired and unpaired long delay (1000-ms) eyeblink conditioning</td>
</tr>
</tbody>
</table>
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Abstract

Behavioral inhibition is a risk factor for the development of anxiety disorders, yet the neural substrates underlying the transition to clinical anxiety are unknown. Increasingly, the cerebellum is gaining recognition for its role in non-motor processes including executive functions such as avoidance and emotion. However, no work to date has examined the cerebellum as a possible neural substrate underlying behavioral inhibition. In the present work, a stress-diathesis approach is utilized to examine behavioral inhibition as a factor contributing to increased vulnerability to anxiety disorders. Inhibited individuals demonstrate facilitated acquisition in cerebellar-dependent associative learning tasks, increased cerebellar connectivity with executive intrinsic connectivity networks, and individual differences in reactivity to visual presentations of faces and scenes, suggesting a cerebellar role in anxiety vulnerability. These findings are placed within the context of cerebro-cerebellar circuitry in an attempt to understand how behavioral inhibition may modulate behavioral and neural reactivity in response to the environment, influencing the development of anxiety disorders.
Introduction

I. A diathesis-stress approach: Role of behavioral inhibition

Increased appreciation of the complexities of mental illness indicates that anxiety disorders are the result of a combination of factors such as sex, genetics and biology [1]. Another factor linked to the development of anxiety is temperament, a core feature of personality, that is evident early in childhood and remains stable throughout the lifespan [2]. Accordingly, low success rates in the treatment of anxiety disorders support that temperament is an essential risk factor in anxiety, as successful treatment would require the alteration of stable character traits [3, 4].

The development of affective disorders is strongly correlated with temperament, especially in respect to behaviorally inhibited temperament (BI;[5-8]). BI is a temperamental construct characterized by avoidance, withdrawal, or apprehension in response to novel people or events [9-13]. Rather than study the broad construct of temperament as a whole, focusing on BI provides a more focused understanding of the interaction between personality and the environment as it concentrates on behavioral responses and physiological reactivity.

The characterization of inhibited temperament is largely due to the longitudinal work of Kagan and colleagues. By studying individual differences in infants 21-22 months old reported as shy (inhibited) or sociable (uninhibited) by their mothers, Kagan and colleagues found consistencies in their behavioral responses. In particular, inhibited infants demonstrated long latencies to interact with unfamiliar people or objects, retreated from novel stimuli, and remained in close contact with their mothers. This behavior significantly correlated between sessions (3-5 weeks apart), reflecting stability over time in classifications of inhibited and uninhibited children.
Follow-up studies demonstrated the stability of behavioral inhibition through childhood and even adolescence, further demonstrating a relationship between those classified as inhibited with greater avoidance of social interactions, and a higher prevalence of clinical anxiety disorders [6-8, 14]. Additionally, inhibited temperament is a heritable trait [15]. Children classified as inhibited are more likely to have parents or siblings diagnosed with clinical anxiety disorders, social phobia, avoidant and over anxious disorders compared to families of uninhibited children [14, 16].

Kagan and colleagues provided invaluable knowledge of the behavioral and physiological profile of behavioral inhibition and its development over time. However, the use of longitudinal assessment is often impractical. Rather, cross-sectional methods using self-report measures provide a convenient means to examine the influence of temperament on behavior. Self-report measures benefit from ease of administration, simple instructions, and time-savings. As such, multiple measures have been constructed to measure both current and prior (childhood) expression of behavioral inhibition.

Early work from Spielberger and colleagues contributed the State-Trait Anxiety Inventory (STAI);[17]. The STAI benefits from a short form, extensive validation, and widespread use. Although not a direct measure of behavioral inhibition, it has often been used in validation of other self-report measures of behavioral inhibition. The Trait anxiety portion of the scale is similar to behavioral inhibition in that it is designed to measure a stable personality characteristic, while State anxiety measures the fluid aspect of mood and emotion by asking questions about the current emotional state.
The Concurrent and Retrospective Self-Report of Inhibition (CSRI and RSRI, respectively; [18]) was designed specifically to measure behavioral inhibition. The CSRI and RSRI use a broad approach to measure the construct of BI through a direct method of questioning. Items for the CSRI and RSRI were gathered from sources such as the Minnesota Multiphasic Personality Inventory [19] and reflect physical symptoms, fear, assertiveness and experiencing anxiety.

In contrast, the Adult and Retrospective Measure of Behavioural Inhibition (AMBI/RMBI; [20]) was developed recently to concentrate on the presence of avoidance in response to new stimuli or social situations. Questions on the AMBI and RMBI are more behavioral in nature, with items generated to capture the construct of avoidance in behavioral inhibition.

These measures are attempting to measure the same underlying construct by different approaches. It is presently unclear which measure is best at differentiating individuals to successfully evaluate individual differences between groups.

II. The Cerebellum: Structure and Function

The cerebellum accounts for approximately 10% of the total brain volume and contains nearly half of all the neurons in the brain. It is highly organized with a distinct topography, cellular structure, and inputs and outputs. The cerebellum is made up of two hemispheres that are structural mirror images and are connected medially by the vermis. Each hemisphere is made up of an outer region of gray matter (the cortex), an inner region of white matter, and three pairs of deep nuclei responsible for cerebellar output; the dentate, the fastigial, and the interposed. In addition to the nuclei, the cerebellum is segregated into sections for anatomical specificity: Lobules I-X, Crus 1 and Crus 2 [21, 22].
Traditionally, the cerebellum is viewed as a motor comparator. The cerebellum is connected with the cerebral cortex as well as multiple subcortical structures including the vestibular nuclei and basal ganglia. Muscle movement, especially coordinated and smooth movements, is the product of a feedback loop between the cerebellum and frontal cortex. Essentially, a “copy” of motor demands is carried from the premotor and motor cortex to the cerebellum via the cerebrocerebellar circuitry. Input projections from the cerebral cortex first synapse at the ipsilateral pons before crossing to the contralateral cerebellar cortex. Output projections synapse at the dentate nucleus, before crossing to the contralateral thalamus and finally back to the cerebral cortex. It is via these cerebro-cerebellar pathways that the cerebellum receives motor demands from the cortex, and then compares feedback from the muscle spindles, joints, and tendons to modify motor behavior, maintain coordination, and perform skilled movements.

The cerebellum contains a highly organized cellular circuitry comprised of both excitatory and inhibitory connections, packed into three layers in the cerebellar cortex. As outlined above, input from the cerebral cortex is carried via the pontine nuclei. A unique quality of the cerebellum is that it is an inhibitory structure. The GABAergic Purkinje cells synapse on the deep cerebellar nuclei to inhibit output. In the case of the cerebrocerebellar circuitry, inhibition of the dentate nucleus of the cerebellum via Purkinje cells modulates output to the thalamus, and finally the cerebral cortex. It is via the unique cellular structure and reciprocal connectivity with the cerebral cortex that the cerebellum modifies motor behavior.

III. Classical Eyeblink Conditioning

Although the cerebellum has long been acknowledged for its role in motor behavior, learning and memory was assumed to be localized in higher cortical
regions. Over the past thirty years, Thompson and colleagues have painstakingly outlined the intrinsic circuitry for a simple form of associative learning, eyeblink classical conditioning, demonstrating the essential role of the cerebellum (for a review see: [23]).

Eyeblink conditioning is based on a simple reflex pathway. In standard delay eyeblink conditioning a tone conditioned stimulus (CS) precedes and co-terminates with a corneal airpuff unconditional stimulus (US) that elicits an unconditional response (UR). Following repeated presentations, the CS gives rise to a conditioned response (CR), which precedes and significantly modifies the US.

Damage to the cerebellar cortex, nuclei, or major afferent pathways abolish or impair acquisition of the CR during eyeblink conditioning. Using rats and rabbits, the essential pathway of eyeblink conditioning has been reduced to two pathways that converge in the cerebellum. Figure 1 provides a simplified schematic of the eyeblink conditioning pathway. Auditory information is transmitted via the pontine nuclei to the cerebellar cortex and interpositus nucleus via mossy fiber connections. From the trigeminal nucleus the US is separated into two pathways: the reflexive route bypasses the cerebellum to produce the reflexive eyeblink UR. The learning route is carried to the cerebellum via the inferior olive. The cerebellar cortex and anterior interpositus nucleus then combines the CS and US streams of information. It is here where the memory trace is stored by altering the firing patterns of Purkinje cells during the development of the CR [24, 25].
In addition to an advanced understanding of its neural substrates, eyeblink conditioning is a simple and sensitive research tool that is amenable to cross species comparisons, is observable from infants to the elderly, and is useful in examining learning in clinical and non-clinical populations. Although acquisition of the eyeblink conditioned response is a relatively simple type of learning, it provides valuable information about how an organism interacts with its environment and indicates the modification of behavior over time.

Converging evidence also indicates that classical conditioning is an essential component in anxiety etiology [1]. In studies specifically measuring eyeblink acquisition, differences have been observed in individuals exhibiting anxiousness and avoidant behaviors. Enhanced acquisition of the conditioned response has been documented in classic studies assessing a form of trait anxiety. In their studies,
Spence and colleagues separated healthy college-aged individuals into high and low anxious groups and then compared acquisition in eyeblink classical conditioning. They found that those who scored high on self-reported trait anxiety demonstrated more conditioned responses than those with low scores [26, 27]. The authors interpreted these differences in terms of “emotionally based drive”, suggesting that anxious participants have increased drive to detect stimuli and produce responses necessary for conditioning to occur.

These studies demonstrated very interesting and important individual differences in eyeblink acquisition. However, very little research work has occurred since to characterize the basis of these differences and under what circumstances they thrive or deteriorate. Recent work using behavioral inhibition to assess individual differences in eyeblink conditioning indicates a relationship between inhibited temperament and associative learning [28, 29]. These findings suggest that patterns of associative learning and avoidance acquisition in anxiety vulnerable populations represent an important and relatively unexplored area of research that may shed light on the essential underlying neural substrates mediating risk for anxiety.

IV. Higher Cognitive Processes of the Cerebellum

A shift in the understanding of the cerebellum indicates it is responsible for functional roles beyond the planning and execution of movements. Recent assessments of its cognitive capacities have demonstrated that the majority of the function of the human cerebellum is associated with cerebral networks involved in non-motor cognitive functions.

The reciprocal anatomical pathways of the cerebral cortex and cerebellum have been previously outlined in terms of motor behavior. It is via these same
pathways that the cerebellum may modulate non-motor cognitive processes. Neuroimaging studies report cerebellar activation during paradigms intended to measure sensory processing, attention, verbal working memory, and emotion [30-37]. Furthermore, an early high-resolution fMRI study revealed specific dentate nucleus activity in response to a cognitive processing task [38]. Although the list of publications specifically assessing cerebellar contributions to higher cognitive processes is short, many other studies also report significant cerebellar activity while leaving interpretation up to the reader. Together, these findings point to an indisputable role of the cerebellum in non-motor processes.

Early observations from neuroimaging are supported by recent breakthroughs in anatomical tract tracing techniques. Transneuronal tracers are viruses that can map multiple synapses in a circuit. For example, a tracer can be injected into specific areas of the brain and then later examined for the presence of neurons labeled by the tracer, indicating anatomical connections. Using this technique in primates, Strick and colleagues have demonstrated both input and output projections between the cerebellum and non-motor regions of the frontal cortex [39-41]. Their first critical finding demonstrated that the dentate nucleus had output projections to non-motor cortical regions, similar to that observed in functional magnetic resonance imagery (fMRI) of executive processes [38]. More recently, they have used viral tracing to understand the organization of cerebellar input and output, such that that a large region of the cerebellum Crus 1 and Crus 2 maintains a closed-loop circuit with the prefrontal cortex area 46 [40]. Together, observations from viral tracing methods reveal that the cerebellum has the necessary anatomical substrates to contribute to higher cognitive processes.
Recent research using resting-state fMRI imaging techniques support anatomical studies in primates. Resting-state imaging methods are based on the observation that neural activity fluctuates, even when the individual is not actively performing a task. By assessing these fluctuations, researchers have noted that certain regions correlate with each other, reflecting intrinsic functional networks of the brain [42, 43]. For example, regions associated with motor function tend to correlate in their activations, waxing and waning together, and are therefore considered to form a particular network [44, 45]. Continued research has demonstrated several intrinsic connectivity networks (ICNs) in the brain corresponding to essential functions like vision, sensorimotor, language and executive function [43-50].

Only recently has the cerebellum received greater attention in terms of connectivity to non-motor ICNs. Habas and colleagues [51] provided one of the first publications exploring cerebellar contributions to the major ICNs. Using independent component analysis (ICA) they assessed cerebellar connectivity to the sensorimotor network, default mode network, executive network and salience network. Critically, their work showed that not only does intrinsic activity of the cerebellum correlate with each network, but that distinct regions of the cerebellum contribute to specific networks. O'Reilly et al. [52] extended resting-state analyses from the general ICA approach to a more specific mask approach - targeting prefrontal, motor, auditory, somatosensory, and visual areas of the brain. Their findings suggest that some regions of the cerebellum may contribute to both motor and non-motor processes (e.g., lobule VI), whereas other regions appear to contribute solely to non-motor networks (e.g., Crus I and Crus II).
In addition to basic research investigations, resting-state fMRI is a useful platform to study clinical and non-clinical individual differences. Resting-state connectivity analyses are a powerful tool in understanding pathologies such as alzheimer’s disease and traumatic brain injury [53, 54]. Additionally, researchers have assessed psychopathologies such as anxiety, demonstrating differences in intrinsic connectivity in a variety of clinical populations including social anxiety disorder [55-57], obsessive compulsive disorder [58], and post traumatic stress disorder (PTSD); [59-62]. Presently, there are no studies assessing individual differences in intrinsic connectivity of non-clinical anxiety vulnerable individuals using resting-state fMRI. Clearly, an examination of intrinsic connectivity differences in anxiety vulnerability will provide important understanding of the neural substrates and processes involved in the development of anxiety disorders.

V. Competing Hypotheses: Neural Substrates Underlying Behavioral Inhibition

Recent work using cerebellar-dependent delay eyeblink classical conditioning indicates a relationship between behavioral inhibition and associative learning [28, 29, 63]. Combined with a recent task-based neuroimaging study demonstrating individual differences in cerebellar reactivity to novel face stimuli in behaviorally inhibited, but not non-inhibited individuals [37], it appears that individual differences in cerebellar functioning may play a role in behavioral inhibition and, as such, anxiety vulnerability.

Although very little research has been dedicated to the cerebellar role in anxiety vulnerability, continued research assesses other regions as essential neural substrates. The hippocampal formation, known for its essential role in learning and memory, is another region that may play a role in anxiety vulnerability. Following indications that individuals with PTSD had smaller hippocampal volumes, Gilbertson
et al., [64] designed an elegant study to test if the reduction in hippocampal volume was a risk factor for or a result of PTSD. By comparing the hippocampal volume of combat exposed veterans with PTSD to their twin with no combat exposure, Gilbertson et al. found that the reduced hippocampal volume was present in both groups. Furthermore, those without PTSD showed greater hippocampal volume, regardless of combat exposure. Together, these findings suggest that reduced hippocampal volume is not an outcome of PTSD, but rather a pre-existing vulnerability to develop PTSD.

Additional support for a hippocampal role in anxiety vulnerability comes from a recent study specifically assessing performance on a computer-based task using measures of behavioral inhibition. Using a probabilistic classification task, researchers found that those scoring high on the AMBI demonstrated faster associative learning as well as significantly greater avoidant responses to stimuli that had the greater probability of being punished (point loss) than those that were rewarded [65].

Clearly, there is support for both hippocampal and cerebellar influence in behavioral inhibition. Further research is necessary to explore the role of these regions and individual differences in functioning as it relates to behavioral inhibition.

**VI. Rationale**

Although disparate sources of information suggest that the cerebellum plays an important role in behavioral inhibition, it is still unclear what the nature of this relationship is. Previous research demonstrates facilitated acquisition in those scoring high on RMBI [28] and AMBI [29,63] in eyeblink classical conditioning. Unfortunately, there are multiple measures of behavioral inhibition and anxiety vulnerability at use in the literature. At present, it is unclear which measures are the
best at differentiating individual in an associative learning task. Furthermore, the
differences observed so far in eyeblink conditioning used a cerebellar-dependent
standard delay paradigm. Individual differences observed could be due to cerebellar
or hippocampal functioning. Although the cerebellum is essential in eyeblink
conditioning, it is possible that behavioral inhibition is characterized by hippocampal
dysfunction, which may also facilitate delay eyeblink conditioning. While the
hippocampus is not necessary to acquire standard delay eyeblink conditioning, it
influences cerebellar functioning, as illustrated by rat studies reporting that lesioning
the hippocampus results in faster delay eyeblink conditioning [66].

Secondly, neuroimaging work concentrating on the cerebellum largely
overlooks individual differences. To date, no work has systematically evaluated the
relationship between cerebellar connectivity and behavioral inhibition. Thus, it is
possible that cerebellar connectivity reported in previous research is driven by a
subgroup of behaviorally inhibited individuals. Examining the patterns of behavioral
responses, connectivity, and reactivity in inhibited and non-inhibited individuals will
clarify the essential neural substrates and may suggest a larger circuitry underlying
anxiety vulnerability.

VII. Outline of Research Studies

The present series of studies were designed to address specific hypotheses
pertaining to the cerebellar role in behavioral inhibition. The following specific aims
are proposed to:

1. Examine the efficacy of measures of behavioral inhibition (AMBI/RMBI and
CSRI/RSRI) and anxiety vulnerability (STAI State and Trait) to differentiate
acquisition of eyeblink conditioning (published results).
2. Extend classical conditioning literature to adolescent anxiety vulnerability and assess the impact of behavioral inhibition on acquisition in standard delay and long-delay conditioned stimulus contingencies supporting either a cerebellar or hippocampal role in anxiety vulnerability (under review).


4. Assess reactivity to stimuli associated with cognitive processing in inhibited and non-inhibited individuals.
Study 1

Facilitated acquisition of eyeblink conditioning in those vulnerable to anxiety disorders

1,2 Meghan D. Caulfield, 2,3 J. Devin McAuley, and 1,2 Richard J. Servatius

1 Graduate School of Biomedical Sciences, University of Medicine and Dentistry of New Jersey, Newark, NJ, USA; 2 New Jersey Medical School, Stress and Motivated Behavior Institute, University of Medicine and Dentistry of New Jersey, Newark, NJ, USA; 3 Department of Psychology, Michigan State University, East Lansing, MI, USA

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Abstract

Behavioral inhibition (BI) increases vulnerability to develop anxiety disorders and is typified by avoidance and withdrawal from novel objects, people, and situations. The present study considered the relationship between BI and temperamental risk factors, such as trait anxiety and acquisition rate of a classically conditioned eyeblink response. One-hundred seventy-four healthy undergraduate students (mean age 20.3 years, 71.8% female) were given the State-Trait Anxiety Inventory and a battery of self-report measures of BI consisting of the Adult and Retrospective Measures of Behavioral Inhibition (AMBI/RMBI) and the Concurrent and Retrospective Self Report of Inhibition (CSRI/RSRI). Participants then underwent standard delay classical eyeblink conditioning consisting of 45 trials with a 500-ms CS overlapping and co-terminating with a 10-ms airpuff US. Individuals with higher scores on the AMBI and Trait Anxiety Inventory, but not the other measures, showed faster acquisition of a conditioned eyeblink response than individuals with lower scores. Results support a relationship between facilitated acquisition of inter-stimulus relationships and risk for anxiety, and suggest that some measures assessing anxiety vulnerability better capture this relationship than others.
1. Introduction

One quarter of the US population is estimated to develop an anxiety disorder at some time in their lives [67, 68]. Another way to look at this statistic is that 75% of Americans do not develop clinical anxiety, raising the question of what is it about an individual that makes them more or less likely to develop an anxiety disorder? So far, it appears that there is no single factor that increases one’s risk for developing an anxiety disorder. Rather, anxiety disorders are best represented by a combination of pre-existing factors that reflect enhanced vulnerability to anxiety, following the experience of stressors in the environment. A stress-diathesis model for the development of anxiety disorders emphasizes changes in stress reactivity following the convergence of a variety of factors such as genetics, biology, sex, personality, and prior experience [1]. While all of these factors require associations between the environment and stressors, present diathesis models of anxiety vulnerability do not take into account individual differences in learning.

Behaviorally inhibited temperament is a personality risk factor linked to increased likelihood to develop anxiety disorders [5-8]. Behaviorally inhibited individuals demonstrate similar behavioral and physiological profiles as seen in clinical anxiety including withdrawal, apprehension, slow latency to approach unfamiliar people or objects [9, 10], altered adrenocortical activity [11], reduced heart rate variability and increased bradycardic responses [12, 13].

Avoidance is the core feature of both clinical anxiety and behavioral inhibition [13, 69-71]. As such, understanding the role of avoidance in the development and maintenance of anxiety is essential. Avoidance is a learned response that is acquired and reinforced over time. Rather than deal with uncontrollable events, anxious individuals assert control by substituting other negative thoughts or feelings.
that are avoidable, providing a feeling of control and temporary relief while at the same time increasing the aversiveness of the undesired stimulus or state in the future, ensuring continued avoidant behavior [1]. Over time, avoidant behaviors become pervasive and uncontrollable such that normal functioning becomes impossible. Because avoidance is a learned process it is possible to measure the acquisition of negative reinforcement contingencies. Individual differences in the speed of acquisition or strength of associations in avoidance may contribute to vulnerability or resiliency. Certain individuals may be more susceptible to acquire and repeatedly express avoidant behaviors, such as those who are behaviorally inhibited, leading to the development of behavioral and cognitive avoidance symptoms associated with clinical anxiety.

Multiple processes underlie avoidance acquisition and maintenance such as sensitivity to acquire inter-stimulus associations and rigidity of expression making it difficult to sift out the essential factors leading to anxiety disorders. One possibility is that increased sensitivity to cues and contingencies in the environment are learned faster in anxious individuals, resulting in better performance on avoidance tasks [65]. Eyeblink classical conditioning provides a means to measure these associations, enabling multiple measures to be taken into account including reactivity, acquisition of the relationship between stimuli, and the rate of extinction. Rather than using operant avoidance paradigms, eyeblink conditioning is a simple and sensitive tool that benefits from an advanced understanding of the neural substrates, amenability for cross species comparisons, control over the stimulus parameters and measurability of multiple aspects of the response. The neural substrates underlying eyeblink conditioning has been documented at length, with converging agreement that the cerebellum is both necessary and sufficient to acquire delay-type eyeblink
Eyeblink conditioning is a measure of associative learning that utilizes a simple reflex pathway. In delay-type eyeblink conditioning, a tone conditioned stimulus (CS) precedes and co-terminates with a corneal airpuff unconditional stimulus (US) that elicits an unconditional response (UR). Over repeated pairings, the CS induces a conditioned response (CR) that precedes and modifies the US.

Differences in acquisition of conditioned eyeblink responses has been demonstrated in individuals demonstrating anxiousness and avoidant behaviors including anxiety [76-79] and BI [28]. Spence and colleagues [26, 27] initiated research on the relationship between anxiousness in healthy individuals and associative learning. Using the Manifest Anxiety Scale (MAS [80]), they separated healthy college-aged individuals into high and low anxious groups and then compared acquisition in eyeblink classical conditioning. In a series of studies, Spence and others found that those who scored high on the MAS demonstrated more conditioned responses (CRs) than those with low scores [26, 27]. Recently, using a similar scale of Trait Anxiety [17], Holloway et al. [76] demonstrated facilitated acquisition as well as proactive interference in Trait anxiety with pre-exposures of the US attenuating learning to a greater degree in high Trait anxious individuals, suggesting those vulnerable to anxiety interpret stimuli in their environment differently. Recently, Myers et al., [28] demonstrated facilitated delay eyeblink acquisition in veterans not reporting current severe post traumatic stress symptoms with high scores on the Retrospective Measure of Behavioural Inhibition [20] compared to low scoring individuals, indicating a relationship between behaviorally inhibited temperament and associative learning.
Parallels are evident between rat models of anxiety vulnerable temperament and humans with self-reported inhibited temperament, suggesting a common neural substrate. Similar to the behaviorally inhibited personality profile, the Wistar-Kyoto rat (WKY) demonstrates inherent anxiousness, vulnerability to stress, and avoidant behaviors [81-88]. WKY male rats acquire eyeblink conditioning significantly faster than outbred Sprague-Dawley rats, with greater asymptotic performance and resistance to extinction [89].

Considering the close relationship between associative learning of cues as predictors of aversive events, enhanced classical conditioning would also be reflected in sensitivity to acquire avoidance responses. Presently, only the Myers [28] study assessed this relationship in terms of behavioral inhibition. While the veterans used were considered healthy in that they did not demonstrate post traumatic stress symptoms, it remains that the experiences of a veteran are likely very different from that of civilians. Therefore, it is important to understand how behavioral inhibition relates to associative learning in other healthy populations.

The current study assessed the relationship between behavioral inhibition and acquisition in delay eyeblink classical conditioning. To approach anxiety disorders from a vulnerability perspective we chose to use a healthy sample of college-aged individuals that minimizes present and past psychopathologies. It is important to note that while we utilized measures of anxiety vulnerability in this study, we did not conduct a structured clinical interview. Therefore, it is possible that some participants may suffer from undiagnosed anxiety disorders.

It is presently unclear which measures are effective in differentiating eyeblink acquisition of healthy individuals. Therefore, multiple measures of behavioral inhibition, the Adult Measure of Behavioural Inhibition (AMBI), the Retrospective
Measure of Behavioural Inhibition (RMBI), the Concurrent Self Report of Inhibition (CSRI), and the Retrospective Self Report of Inhibition (RSRI) were used. Additionally, instead of using the MAS, which was designed specifically to separate individuals in experimental studies, we chose to use the State-Trait Anxiety Inventory (STAI-Y), which is similar in its approach but benefits from extensive validation and widespread use. Furthermore, assessing behavioral inhibition in addition to trait anxiety allowed evaluation of facilitated associative learning in terms of specific constructs, such as behavioral inhibition, or a general over-arching principle, such as anxiousness.

We assessed the effectiveness of these measures in separating eyeblink acquisition (as determined by the number of CRs) in high and low scoring individuals. Following eyeblink acquisition, participants received a series of CS-alone trials allowing assessment of the relationship between extinction and high and low scorers on each measurement. We hypothesized that high scoring individuals would acquire delay eyeblink conditioning faster than low scorers. Specifically, given the relationship between anxiety, avoidance, and associative learning we expected the AMBI/RMBI, which emphasizes avoidant behaviors to be the best at differentiating learning. Furthermore, given that anxiety vulnerability is a stable, long-term risk factor, we expected that STAI-Trait would differentiate learning, but not STAI-State, which is a measure of transient, temporary anxious feelings in the present.

2. Methods
2.1 Participants

One-hundred seventy-four students (n = 125 female, n = 49 male), ages 18 – 40 years (M = 20.3, SD = 2.8), from a large Midwestern university participated in
return for partial credit in an undergraduate psychology course. All study materials were reviewed and approved by internal review and informed consent was obtained from all participants prior to any experimental procedures.

2.2 Self-report measures.

Participants completed a battery of self-report questionnaires prior to undergoing eyeblink conditioning. Participants were given the Adult and Retrospective Measure of Behavioural Inhibition (AMBI/RMBI; [20]), the Concurrent and Retrospective Measures of Behavioral Inhibition (CSRI/RSRI;[18]) and the Spielberger State/Trait Anxiety Inventory (STAI;[17]).

The Adult Measure of Behavioural Inhibition is a 16-item self-report measure that assesses the presence of inhibition or avoidance in response to new stimuli or social situations. Items ask questions such as “Do you tend to withdraw and retreat from those around you?”, or “Do you tend to introduce yourself to new people?” to assess 4 underlying constructs of fearful inhibition, risk avoidance, non-approach and low sociability. Participants are asked to respond to questions on a three-point scale and indicate no/hardly ever (“0”), some of the time (“1”), and yes/most of the time (“2”). Total scores can range from 0 to 32. Similarly, the Retrospective Measure of Behavioural Inhibition is an 18-item self-report measure on the same scale of 0-2 that assesses childhood memories (during elementary school) of responding in unfamiliar situations. Total scores can range from 0-36. The scales demonstrate reliability with no differences in test-retest scores, and significant (p<.001) discriminant validity in separating anxiety, depression and control groups [20]. Our sample demonstrated high internal consistency with Cronbach’s alpha of .78 for AMBI and .86 for RMBI.
The Concurrent and Retrospective Self-Reports of Inhibition are similar to the AMBI/RMBI in that it measures behaviors consistent with behavioral inhibition especially in regards to withdrawal in social situations. The CSRI/RSRI is broader in its approach and utilizes a more direct method of questioning. Questions are answered on a 5-point scale with answers specific to the question wording (e.g., ranging from “0-4 days” to “more than 20 days” or from “never” to “very often”) but always going from least to most inhibited. The CSRI asks 31 self-report questions on the 5-point scale reflecting four aspects of behavioral inhibition including fears, behaviors that reflect fear, behaviors that express assertiveness and experiencing anxiety. Total scores on the CSRI can range from 31 to 155. Similar to the RMBI, the RSRI asks participants 30 self-report questions on the 5-point scale with total scores ranging from 30-150 about childhood experiences relating to the construct of behavioral inhibition as demonstrated by two factors of school/social (“during recess, did you play with the main group of children?”) and fear/illness (“How often did you have nightmares?”). Some questions did not load on any specific factor but are still part of the measure [18]. Both measures demonstrated high internal consistency with Cronbach’s alpha of .82 for CSRI and .83 for RSRI.

The Spielberger State/Trait Anxiety Inventory is a 40-item self-report questionnaire with responses ranging from 1 (“almost never”) to 4 (“almost always”) with total scores ranging from 40 to 160. The STAI is separated into two parts, State and Trait anxiety, each consisting of 20 questions: State Anxiety is assumed to change with mood and emotion and asks questions about the current emotional state of the participant such as “I am tense” and “I feel at ease”. Trait anxiety is a relatively stable personality characteristic and asks questions about general feelings and behaviors such as “I feel nervous and restless” and “I feel satisfied with myself”
Both measures demonstrated high internal consistency with Cronbach’s alpha of .93 for STAI-State and .88 for STAI-Trait.

2.3 Eyeblink conditioning

Eyeblink conditioning apparatus and procedures were previously described [90]. Briefly, participants wore a customized David Clark aviation headset (Worcester, MA) from which auditory (tone) stimuli produced by signal generators (LabVIEW, National Instruments, Austin, TX) and a digital to analog converter (PCI-604E, National Instruments, Austin TX) were delivered. Sound levels were verified and checked for consistency with a Realistic sound meter (Radio Shack). The conditioned stimulus was an 82 dB 1200 Hz pure tone 500 ms in length. The headphones were also fitted with a boom placed 1 cm from the cornea that delivered a 5 psi airpuff US via sylastic tubing connected to a regulator and released by a computer controlled solenoid valve (Clipper Instruments, Cincinnati, OH). To record eyeblink responses, participants are fitted with three silver/silver chloride electromyography (EMG) electrodes covered in conductive gel. Two EMG electrodes are placed above and below the right eye and the third is placed on the neck as the ground electrode. The signal is passed to an isolated physiological amplifier (UFI, Morro Bay, CA) and band-pass filtered for low 10 Hz and high .1 Hz frequencies and amplified by 1000. The signal was sampled at 200 Hz by an analog to digital board (PCI-604E, National Instruments, Austin TX). Each session lasted approximately 40 minutes, during which participants watched a silent movie (Toy Story) to reduce boredom and help maintain a forward-facing gaze.

2.4 Procedure

All participants received the same battery of questionnaires (AMBI/RMBI, CSRI/RSRI and STAI State and Trait) followed by the delay-type classical
conditioning. Following consent and the completion of questionnaires, subjects were fitted with EMG electrodes, the signal quality was checked and conditioning began. Initially, each participant was exposed to three US alone stimuli to establish appropriate responses to the airpuff and measure the UR prior to conditioning. Participants were conditioned with a delay procedure consisting of 45 CS-US paired trials (500-ms, 83-dB 800 Hz pure tone CS co-terminating with a 50-ms airpuff US) and 15 CS-alone trials consisting only of the 500-ms pure tone. Trials were separated by an inter-trial interval ranging from 25-37 s ($M = 30$ s). The duration of the entire experimental session was one hour with eyeblink conditioning lasting approximately 40 minutes.

2.5 Eyeblink Data Processing

For all sessions, eyelid EMG recordings were evaluated for each participant on a trial-by-trial basis. Sessions with excessive signal noise (loss of more than 10% of trials) or that demonstrated a lack of a UR were discarded and not used for further analysis. To be recorded as an eyeblink the smoothed signal must change by more than the mean activity plus 4 times the standard deviation in a 125-ms comparator window. Responses meeting this criterion and occurring within 200 ms of CS onset are scored as an $\alpha$-response or orienting response, those between 200 ms after CS onset but prior to US onset are considered a CR and those occurring in response to the US are considered an UR [90].

2.6 Data Analysis

For eyeblink conditioning, the dependent measure was percent CRs within a block of trials. Repeated-measures ANOVA with within-subjects factor of block and between-subjects factors of high and low scorers based on a median split of the collected sample on AMBI/RMBI, CSRI/RSRI, and State/Trait. Nine blocks
consisting of 5 trials each was used to assess acquisition and three blocks of 5 trials was used to assess extinction.

3. Results

3.1 Self-report measures of anxiety vulnerability

Mean scores for all of the self-report measures with standard deviations are shown in Table 1 separated by sex. A point-biserial correlation demonstrated no relationship between sex and any of the self-report measures (all \( p \)'s > .299). Correlations between survey measures are shown in Table 2; statistical significance was determined using Bonferroni corrected \( p \) value of .007. Correlations between survey measures were all positive. Notably, both adult measures of BI (AMBI and CSRI) and childhood measures (RMBI and RSRI) were more strongly correlated than the adult measures were correlated with the child measures. Additionally, the STAI-Trait was reliably correlated with all measures of BI, especially the adult measures, while the STAI-State did not correlate with the AMBI or RMBI.

3.2 Eyeblink conditioning

Analysis of eyeblink conditioning data was completed for 117 participants; data from the remaining 58 were unusable due to poor signal quality, inability to stay alert throughout the 40 minute session, or failure to exhibit the unconditioned response\(^1\). The distribution of males and female participants included in eyeblink analysis did not differ from the distribution of male and female participants who had to be excluded, \( X^2(1, N=170) = .21, \ p = .64 \). Included and excluded participants also did not differ in their survey scores (all \( p \)'s > .08 using independent samples t-tests). For the 117 participants included in the eyeblink analysis the average age was 19.9

\(^1\) A 33% loss of data in this eyeblink conditioning paradigm is within the normal range (Holloway et al., 2012; Myers et al., 2011).
years (SD = 1.7, range = 18-26 years) with 36 males and 81 females (69.2% female) and 14.0 years of education (SD = 1.4, range = 11-17 years). A repeated measures ANOVA of percent CRs revealed a significant main effect of training block, F(8,928) = 5.879, MSE = .05, \( p < .001 \), with visual inspection of the learning curves showing increasing acquisition of the CR throughout the training period. A repeated measures ANOVA to assess extinction over the three blocks revealed a significant effect, F(2,232)=8.125, MSE = .05, \( p < .001 \), with fewer CRs in later extinction blocks.

3.3 Self-report measures and eyeblink acquisition

For subsequent analyses, we compared the ability of the different self-report measures to differentiate fast and slow learners by first performing a median split on each measure to create a high scoring group and a low scoring group and then comparing acquisition for the two groups. We chose this approach for the following reasons: First, there are no published cutoffs defining those at risk for anxiety for the AMBI/RMBI, CSRI/RSRI or STAI and this method afforded a conservative approach that would facilitate comparisons across surveys. Second, the approach of using a median split is the same approach that was used to group individuals during discriminability assessments of these scales during their validation. Average scores for the high and low scoring groups for each survey are shown in Table 3.

Independent samples t-tests comparing the amplitude of the unconditioned response during trials in which only the US was presented revealed no significant differences between groups, but a correlation between UR amplitude and overall

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2 Median splits were calculated using the upper real limit of the median score of the sample. For example, if the median is 11, the upper real limit is 11.5, with those greater classified as “high” and those scoring less classified as “low”.
acquisition was significant, \( r = .343, p < .001 \). Consequently, UR amplitude was included as a covariate for the all of the remaining analyses.

Using a hypothesis-driven stepwise approach we used three separate ANOVA comparisons for each set of measures. We first assessed individual differences in acquisition for the AMBI and RMBI measures. Our expectation was that AMBI and RMBI would show significant differences in learning. A 2 (Group: high, low) x 9 (Block) mixed measures ANOVA revealed a significant interaction following Bonferroni correction of .025 between group and block, \( F(8,912)=2.401, p= .014 \) for the AMBI measure but not RMBI measure, all p's > .468 (Figure 1). We next tested to see if the widely used BI measures of CSRI and RSRI were able to differentiate learning as well as AMBI. A 2 (Group: high, low) x 9 (Block) mixed measures ANOVA showed no significant differences in learning for groups created using the CSRI and RSRI, all p's > .207. We then assessed learning differences in groups created using the STAI-Y measures of State and Trait anxiety. A 2 (Group: high, low) x 9 (Block) mixed measures ANOVA demonstrated a significant interaction between group and block, \( F(8,912)=3.137, p=.002 \) for the STAI-Trait measure (Figure 1) but not for STAI-State, all p's > .133. Finally, 2 (Group: high, low) x 3 (Block) mixed measures ANOVAs revealed no significant differences between groups in extinction for any of the self-report measures, all p's > .111.

AMBI and Trait were further analyzed to ensure that scores around the median weren't driving learning differences between groups and that the extremes of the measures maintained observed acquisition differences. To assess this, we selected the upper and lower 1/3 on the AMBI and Trait measures. A 2 (survey score: highest 1/3, lowest 1/3) x 9 (learning block) mixed measures ANOVA with UR amplitude as a covariate indicated a significant interaction, \( F(8,728)=1.961, p=.049 \).
between AMBI and learning. Individual differences in acquisition also remained for the highest and lowest scoring Trait groups, F(8,616)=2.754, p= .005 (Figure 2).

A Spearman's rho correlation was used to further analyze the relationship between scores and the average number of CRs over the entire acquisition session of 45 trials. While AMBI was not significantly correlated with overall acquisition, rs[117] =.037, p =.689, Trait did reveal a significant positive correlation between learning and acquisition rs[117] =.186, p =.045. Furthermore, none of the other measures were significantly correlated with overall acquisition, all p's > .247. Finally, no measures significantly correlated with extinction, all p's > .169.

4. Discussion

The present study assessed the relationship between self-report measures of anxiety vulnerability and acquisition of an associative learning task. In an extension of previous work that demonstrated faster learning in anxiety vulnerable groups, we assessed the effectiveness of measures of anxiety vulnerability to differentiate acquisition in delay-type eyeblink conditioning. We found that while highly intercorrelated, the measures did not equally discriminate between fast and slow learners.

4.1 Anxiety vulnerability and associative learning

The present study demonstrated that individual differences in eyeblink conditioning were related to measures of anxiety vulnerability, such that those scoring high on certain measures, specifically the AMBI and STAI-Trait, acquired eyeblink conditioning faster than low scorers. Group differences between high and low scores were not significant for the other measures examined in this study, suggesting that AMBI and Trait measures may differ in some way from the CSRI/RSRI and RMBI that enables better prediction of associative learning.
Considering that the AMBI and CSRI are both measures of behavioral inhibition, differences in the efficacy for the measures to separate fast and slow learners suggests that there may be fundamental differences in how these measures assess the construct of behavioral inhibition. Comparing the question and answer options for each scale reveals some potential differences. The more direct questioning method of the CSRI/RSRI, with its inclusion of questions about physical symptoms of anxiety and specific frequencies of events may fail to recognize individuals who are behaviorally inhibited but do not manifest overt symptoms of anxiousness.

In this study we demonstrated that those who endorse more AMBI questions acquire eyeblink conditioning faster. In a previous eyeblink conditioning study acquisition differences were observed in comparisons of those with high vs. low RMBI, but not AMBI [28]. One explanation for this inconsistency is sampling differences. The Myers et al. [28] study used a sample of older (M=51.2 years) veterans with previous combat experience, unlike our sample of younger (M=20.5 years) group of college undergraduates. Presumably, these two groups differ in many ways such as personality, motivation, and previous experience that may be reflected in self-report measures.

Another explanation for the effect of AMBI in the present study and RMBI in Myers et al. [28] may be related to a “Do not remember” option that was available to participants in the Myers et al. [28] study. In this case, participants who used this option received pro-rated scores based on answers to other questions on the subscale. It is possible the forced-choice nature of responses in the present study lead participants to reply inappropriately if they did not remember. Demand characteristics and accurate personal historical recall may present other possible explanations for the variations of the present study from the Myers et al., [28]
findings. Even though the study had no clinical bearing, the hospital setting in the Myers et al. [28] study may distort answers to questions in the present compared to the past. A comparison of mean scores in the two samples reveals that veterans’ RMBI scores differed by 4.7 points (from 12.6 on RMBI to 17.2 on AMBI) whereas college students differed only by 0.5 points (from 11.8 on RMBI to 12.3 on AMBI), suggesting that veterans’ responses are less stable between past recall and present. Together, Gladstone & Parker’s [20] behavioral inhibition measures have demonstrated group differences in eyeblink conditioning indicating potential for the AMBI and RMBI to differentiate associative learning. It is difficult to make direct comparisons at present given the few studies and disparate samples. Therefore, the specific role of the AMBI and RMBI remains unclear. While neither alone is the best solution, a combined solution may reveal that those scoring high on combined AMBI and RMBI have significantly more CRs overall and learn faster. Future research will assess both scales and utilize the best questions from each to capture the behaviors underlying enhanced associative learning.

Scores on the STAI-Trait also differentiated individual’s associative learning. Similar to Spence and colleagues [17], participants who scored higher on the Trait measure acquired eyeblink conditioning faster and demonstrated more CRs overall than individuals scoring in the lower median, a difference that remained when comparing the upper and lower 1/3 of scores. This suggests that the MAS and STAI-Trait are measuring similar underlying constructs, although the Trait does it with a shorter form and allows comparisons to be made between a stable, long-term temperament and feelings which are due to a temporary state of anxiousness. This outcome is supported by Holloway et al.’s [76] recent report of facilitated delay eyeblink acquisition following context preconditioning of those scoring high on the
STAI-Trait. As a further step to ensure increased sensitivity to the US (due to state anxiety) was not sufficient to explain differences observed in acquisition we compared the magnitude of the UR and found no significant differences, indicating that increased anxiousness is not responsible for observed differences in eyeblink acquisition.

In this study, associative learning significantly correlated with Trait but not AMBI measures. Learning in eyeblink conditioning is a non-linear and dynamic process that is not the same for all individuals. For this reason, it is difficult to represent eyeblink conditioning with a single value. Overall, those with high scores on AMBI and Trait learn faster, but on an individual basis this may be due to fast learning in the first block, or a maintained high percent of CRs later, making it impossible to represent learning with a single value such as overall acquisition. Additionally, the measures of anxiety vulnerability used here measure the presence of anxiety vulnerability, and not its absence. Thus, a participant can only be described as higher or lower in terms of the presence of behaviorally inhibited behaviors, not if they are behaviorally “uninhibited”, thereby skewing the relationship between low scores and learning. The positive correlations for both measures with overall acquisition suggest that a larger sample or the use of a sample with extreme high and low scores may reveal the relationship between learning and AMBI.

4.2 Risk for anxiety disorders

Individuals can be at-risk to develop anxiety disorders through a variety of vulnerabilities. A diathesis approach stresses the interaction between environment and pre-existing risk factors that increase the likelihood of developing anxiety disorders such as post-traumatic stress disorder [1]. In addition to temperament [16, 17, 43, 91], other risk factors include brain abnormalities [92], genetic polymorphisms
[93, 94], previous stressful experiences [95-97] and sex [98]. Individuals at increased risk for anxiety disorders process the contingencies surrounding events in their environment differently, the outcome of which is increased avoidance – a core feature of anxiety disorders [69]. Here, we extend this approach to suggest the inclusion of aberrant associative learning.

4.3 Limitations and conclusions

Females have also been found to be at greater risk than males for developing anxiety disorders. Furthermore, females demonstrate enhanced acquisition in eyeblink conditioning at times [99]. Even though the sampling of males and females was skewed with over 2/3 female, the present study did not find a significant effect of sex in eyeblink acquisition. Additionally, sex was not significantly correlated with any of the measures of anxiety vulnerability, indicating that sex differences in risk for anxiety are not measurable by the self-report measures used in this study.

The present study was designed to use the standard approach of delay eyeblink conditioning with 100% reinforced trials in acquisition. This design was selected because it is considered the optimal parameters for CR acquisition. An important question for future research will be to understand how schedules of reinforcement and CS duration influence acquisition in anxiety vulnerable individuals.

While self-report benefits from its direct collection of individual’s responses, it suffers a few drawbacks that should be acknowledged. A fundamental issue with all studies using self-report survey measures are their reliability and accuracy. It remains a concern that individuals are not as capable of honestly reporting their behaviors as desired. Retrospective recall is susceptible to time forgetting, time displacement, and distortion. It is possible an individual with behaviorally inhibited tendencies as an adult would conform to that pattern and report similar tendencies
as a child. However, strong correlations between the BI measures suggest resistance to distortion in the present study, but are unable to account for different acquisition patterns between AMBI and RMBI or with the CSRI and RSRI. Future research will have to assess how biases in self-report may relate to associative learning.

Our reliance on self-report measures of anxiety vulnerability also makes the assumption that participants are demonstrating a vulnerability to anxiety disorders in their responses, and not the preclinical manifestation of anxiety disorders. For various reasons including availability and time, this study did not use a structured clinical interview to ascertain if participants are presenting with symptoms congruent with a diagnoses of anxiety disorders. Therefore, some participants may have as-yet undiagnosed anxiety disorders. Future studies could assess the differences between anxiety vulnerability and diagnosed anxiety disorders on associative learning tasks.

This study suggests that associative learning can be differentiated with self-report scales. Here, we demonstrate that the AMBI and Trait are able to separate individuals into faster and slower learning groups. Furthermore, when the criterion was extended to the highest and lowest scoring individuals, AMBI and Trait were able to maintain significant differences between the two groups. It should be noted that the median score of 11 on both the AMBI and RMBI are very low compared to other studies, with medians reported at 16.5 and 13.5 [20], and 14 and 15 in our own studies using New Jersey college students (unpublished observations). These differences may reflect something basic about the way Midwestern students answer questionnaires, about differences in behavior or experiences as asked by the AMBI and RMBI, or about the presence of behavioral inhibition in the sample. Considering
the inconsistency observed between the AMBI and RMBI, future studies would benefit from the use of other samples to assess the reliability and generalizability of these findings and provide a clearer understanding of the dynamic between the AMBI and RMBI measures.

The relationship between measures of anxiety vulnerability and associative learning is important to understand how risk translates to clinical anxiety. As with the vulnerabilities outlined by a diathesis model, facilitated associative learning and temperament (as measured by Trait or AMBI) may be a pre-existing risk factor that provides a pathway to developing anxiety disorders. A consistent and reliable self-report measure of anxiety vulnerability that reflects associative learning would reveal the behaviors, temperament, and underlying constructs responsible for translating risk to diagnosis in anxiety disorders.
Table 1. Descriptive summary of scores on self-report scales

<table>
<thead>
<tr>
<th>Survey</th>
<th>Male (n=49)</th>
<th>Female (n=125)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Raw Score (SD)</td>
<td>Mean Raw Score (SD)</td>
</tr>
<tr>
<td>AMBI</td>
<td>12.9 (4.5)</td>
<td>12.6 (5.0)</td>
</tr>
<tr>
<td>RMBI</td>
<td>12.9 (6.9)</td>
<td>12.2 (6.9)</td>
</tr>
<tr>
<td>CSRI</td>
<td>69.1 (12.0)</td>
<td>72.1 (12.0)</td>
</tr>
<tr>
<td>RSRI</td>
<td>62.5 (12.7)</td>
<td>62.7 (14.2)</td>
</tr>
<tr>
<td>TRAIT</td>
<td>40.2 (8.9)</td>
<td>38.2 (9.0)</td>
</tr>
<tr>
<td>STATE</td>
<td>33.6 (9.0)</td>
<td>35.4 (12.1)</td>
</tr>
</tbody>
</table>
Table 2. Relationship of self-report measures of anxiety vulnerability ($n=174$).

<table>
<thead>
<tr>
<th>Measure</th>
<th>AMBI</th>
<th>RMBI</th>
<th>CSRI</th>
<th>RSRI</th>
<th>TRAIT</th>
<th>STATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMBI</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMBI</td>
<td>0.306**</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSRI</td>
<td>0.615**</td>
<td>0.411**</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSRI</td>
<td>0.274**</td>
<td>0.602**</td>
<td>0.507**</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAIT</td>
<td>0.246**</td>
<td>0.179</td>
<td>0.443**</td>
<td>0.259**</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>STATE</td>
<td>0.138</td>
<td>0.135</td>
<td>0.373**</td>
<td>0.250**</td>
<td>0.349**</td>
<td>-</td>
</tr>
</tbody>
</table>

**Denotes significant correlations, $p < 0.001$.**
Table 3. Summary of eyeblink conditioning groups made for comparison of high and low scores on AMBI, rMBI, CSRI, RSRI, STAITrait and STAI-State (n=117).

<table>
<thead>
<tr>
<th>Survey</th>
<th>Median</th>
<th>High scorers</th>
<th>Low scorers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
<td>Mean raw score ($SD$)</td>
<td>Mean percent score ($SD$)</td>
</tr>
<tr>
<td>AMBI</td>
<td>11.0</td>
<td>16.7 (4.1)</td>
<td>52.1 (12.7)</td>
</tr>
<tr>
<td>RMBI</td>
<td>11.0</td>
<td>17.9 (4.8)</td>
<td>43.9 (13.3)</td>
</tr>
<tr>
<td>CSRI</td>
<td>70.0</td>
<td>80.2 (8.0)</td>
<td>51.7 (5.1)</td>
</tr>
<tr>
<td>RSRI</td>
<td>60.0</td>
<td>71.9 (8.9)</td>
<td>47.9 (5.9)</td>
</tr>
<tr>
<td>TRAIT</td>
<td>37.0</td>
<td>45.3 (7.5)</td>
<td>56.6 (9.4)</td>
</tr>
<tr>
<td>STATE</td>
<td>33.0</td>
<td>42.5 (7.7)</td>
<td>53.2 (9.6)</td>
</tr>
</tbody>
</table>

Both mean and raw percent scores are presented.
SD, standard deviation; AMBI, Adult Measure of Behavioural Inhibition; RMBI, Retrospective Measure of Behavioural Inhibition, CSRI, Concurrent Self Report of Inhibition; RSRI, Retrospective Self Report of Inhibition.
Figure 1. Eyeblink conditioned responding of high and low scoring groups. Significant differences of acquisition of the CR between high and low scoring groups were observed for AMBI, $F(8,912)=2.401$, $p=0.014$, and STAI-Trait, $F(8,912)=3.137$, $p=0.002$. No other measures were able to significantly differentiate learning. Error bars represent the standard error of the mean.
Figure 2. Eyeblink conditioned responding of the upper and lower 1/4 of scores on AMBI and Trait. Significant differences of CR acquisition remain for extreme scoring groups for both the AMBI and Trait measures. Error bars represent the standard error of the mean.
Study 2

Facilitated acquisition of standard but not long delay classical eyeblink conditioning in behaviorally inhibited adolescents

Caulfield, M.D\textsuperscript{a,b,c}, VanMeenen, K.M\textsuperscript{b,c}, Servatius, R.J\textsuperscript{a,b,c}.

\textsuperscript{a}Graduate School of Biomedical Sciences, Rutgers University, Newark, NJ, USA;

\textsuperscript{b}Stress and Motivated Behavior Institute, East Orange, NJ, USA;

\textsuperscript{c}Department of Veterans Affairs, New Jersey Health Care System, East Orange, NJ, USA.

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Abstract

Adolescence is a key age in the development of anxiety disorders. The present study assessed the relationship between behavioral inhibition, a risk factor for anxiety typified by avoidance, and acquisition of the classically conditioned eyeblink response. 168 healthy high school students (mean age 15.7 years, 54% female) were given a battery of self-report measures including the Adult Measure of Behavioural Inhibition (AMBI). The study compared acquisition of three experimental training conditions. Two groups were given paired CS-US training: standard delay of 500-ms or long delay of 1000-ms with CS overlapping and co-terminating with a 10-ms airpuff US. A third group received unpaired training of 1000-ms CS and 10-ms airpuff US. Inhibited individuals showed faster acquisition of the conditioned eyeblink response in the 500-ms CS condition, but not in the paired 1000-ms condition. No differences in spontaneous blinks or reactivity to the stimulus were evident in the 1000-ms unpaired CS condition. Results support a relationship between associative learning and anxiety vulnerability that may be mediated by cerebellar functioning in inhibited individuals.
1. Introduction

Adolescence is a key period for the development of anxiety disorders. With a median age of 11, onset of anxiety occurs much earlier than any other psychiatric illness. Furthermore, half of all lifelong cases of clinical anxiety begin by age 14 [67, 68]. The sensitive period of adolescence provides a unique opportunity to study the development of anxiety disorders. So far, it appears that a combination of vulnerabilities contribute to increased risk for developing clinical anxiety. In this vein, a stress-diathesis model emphasizes that the convergence of such factors including genetics, biology, sex, prior experience and personality alters reactivity to stressors in the environment [1]. Recent research suggests that individual differences in learning may also be an important risk factor for anxiety vulnerability [29, 76, 100].

Behavioral inhibition (BI) is a personality factor linked to the development of anxiety disorders [5-7]. BI is observable early in life and persists through the lifespan [101]. Individuals with BI demonstrate similar physiological and behavioral profiles as those with clinical anxiety including altered heart rate reactivity [12, 13], adrenocortical activity [11], apprehension, withdrawal and avoidance [9, 10].

Avoidance is a key symptom of both behavioral inhibition and clinical anxiety [13, 69, 71], suggesting it is an essential component in the development and maintenance of anxiety. Avoidance is a learned response that is acquired and reinforced over time. As such, avoidance can be measured by assessing acquisition of negative reinforcement contingencies. Those vulnerable to anxiety disorders may be more susceptible to acquire and repeatedly express avoidant behaviors, leading to the avoidant thoughts and behaviors associated with clinical anxiety.
It is still unclear which factors underlying avoidance acquisition are essential in the development of anxiety. One possibility is that anxious individuals are more sensitive to the cues and contingencies in their environments, resulting in faster learning and better performance on avoidance tasks [65]. Another means of measuring these associations is through eyeblink classical conditioning, a well-established and reliable model for understanding human learning. In eyeblink conditioning, a conditioned response (CR) develops after repeated presentations of a conditioned stimulus (CS) and an unconditional stimulus (US). Rather than use operant conditioning methods, eyeblink conditioning benefits from its procedural simplicity and an advanced understanding of the neural substrates underlying response acquisition with general consensus that the cerebellum is both necessary and sufficient to acquire standard delay eyeblink conditioning [25, 72, 75]. Variations of this basic paradigm have shown that rates of acquisition are affected by prior experience with the CS and US (e.g., proactive interference; [76]), by altering the reinforcement schedule [29], or by adjusting the contingencies between the CS and US such as in long delay and trace paradigms.

The effects of development on the acquisition of the conditioned eyeblink response have been assessed at length in infants and adults, largely overlooking the period of adolescence. Using infants and young children researchers have used eyeblink conditioning to demarcate the development of key underlying neural substrates [102-105]. Research in adults concentrates on aging to understand the neurobiology underlying age-related memory disorders [106-109]. Considering that adolescence is a critical period in refining cortical connections as well as for the development of psychopathologies such as anxiety and schizophrenia [110, 111]
understanding how eyeblink conditioning is affected may shed important light on underlying neural networks.

Using eyeblink conditioning we found that college-aged participants who score high on the Adult Measure of Behavioural Inhibition (AMBI), a self-report measure of behaviorally inhibited temperament, demonstrate significantly faster learning in a standard delay (500-ms) conditioning paradigm. At face value, this indicates that there is something fundamentally different about how behaviorally inhibited individuals learn about the basic stimuli in their environments, regardless of valence. However, the underlying processes are still unknown. The purpose of this study was two fold: First, we utilized a basic science approach to assess acquisition of standard delay eyeblink in an adolescent sample for comparisons to other age groups. Second, we addressed two possible theories underlying facilitated learning observed in anxiety vulnerable individuals by comparing acquisition of standard delay (500-ms) to a long delay (1000-ms) CS durations. Longer CS durations have slower ontogenetic development [104] and are more difficult to acquire than standard delay durations [104, 112]. Additionally, long-delay and trace paradigms demonstrate similar learning curves, with a reduction of learning in long delay, and a drastic reduction in trace paradigms following hippocampal lesion in rats [112]. While the hippocampus is not necessary to acquire the CR at 500-ms, it does interfere with cerebellar functioning, as demonstrated by research in rats reporting that lesioning the hippocampus results in faster delay eyeblink conditioning [66]. Therefore, long CS durations provide a useful paradigm to explore the influence of higher cognitive brain regions such as the hippocampus on acquisition without altering the conditioning parameters as drastically as trace conditioning would.
Comparing acquisition at longer CS durations will provide useful information to understand the neural substrates underlying anxiety vulnerability in adolescents. If behaviorally inhibited individuals show faster learning or no differences compared to non-inhibited in the 1000-ms CS duration then support for the cerebellar hypothesis is garnered. If inhibited individuals show a deficit in learning compared to non-inhibited, then this would support the hippocampal theory such that reduced hippocampal interference facilitates learning at 500-ms. At 1000-ms when the hippocampus is necessary to acquire the conditioned response it is unable to meet the demand, resulting in poorer learning.

2. Methods

2.1 Participants

168 participants were recruited from a local public high school in New Jersey. Participants’ ages ranged from 13-19 years (M=15.7, SD=1.25). Parental consent forms were signed prior to participation for all students, as well as informed assent (participants under 18) or informed consent (18 and over) in accordance with procedures approved by the high school and University of Medicine and Dentistry of New Jersey Institutional Review Board.

2.2 Self Report Measures

Participants completed self-report measures including the Adult and Retrospective Measure of Behavioural Inhibition (AMBI/RMBI;[20]), and the State/Trait Anxiety Inventory (STAI [17]).

The Adult and Retrospective Measure of Behavioural Inhibition (AMBI/RMBI [20]) is a self-report measure that assesses inhibition or avoidance in response to new stimuli or social situations. It is reliable and has high discriminant validity in separating anxiety, depression, and control groups [20]. Scores on the 16-item
AMBI range from 0-32 and include questions about current behaviors such as “Do you tend to withdraw and retreat from those around you?”, and “Do you tend to introduce yourself to new people?”. Scores on the 18-item RMBI range from 0-36 and include questions about childhood (during elementary school) behavior.

The Spielberger State/Trait Anxiety Inventory is a 40-item self-report questionnaire with total scores ranging from 40 to 160. The STAII is separated into two parts, State and Trait anxiety, each consisting of 20 questions: State Anxiety is assumed to change with mood and emotion and asks questions about the current emotional state of the participant such as “I am tense” and “I feel at ease”. Trait Anxiety is a relatively stable personality characteristic and asks questions about general feelings and behaviors such as “I feel nervous and restless” and “I feel satisfied with myself” [17].

2.3 Eyeblink conditioning

Eyeblink conditioning apparatus and procedures was the same as previously described [90]. Participants were fitted with a customized David Clark aviation headset (Worcester, MA) that delivered the tone conditioned stimulus and airpuff unconditional stimulus. Auditory stimuli were produced by a signal generator (LabVIEW, National Instruments, Austin, TX) and a digital to analog converter (PCI 6025E, National Instruments, Austin, TX). Sound levels were verified and checked for consistency with a Realistic sound meter (Radio Shack). The conditioned stimulus was an 82dB 1200Hz pure tone 500-ms or 1000-ms in length. A boom on the headphones placed 1 cm from the cornea delivered a 5 psi airpuff US via sylastic tubing connected to a regulator and released by a computer controlled solenoid valve (Clipper Instruments, Cincinnati, OH). To record eyeblink responses, three silver/silver chloride electromyography (EMG) electrodes covered in conductive gel
were placed above and below the right eye and on the neck. The EMG signal is passed to a physiological amplifier (UFI, Morro Bay, CA), band-passed filtered between 1Hz and 30Hz, and amplified by 1000. The signal was sampled at 200 by an analog to digital board (PCI 6025E, National Instruments, Austin, TX) and connected to an IBM computer. Each session lasted approximately 35 minutes, during which participants watched a silent movie (Planet Earth) to reduce boredom and maintain a forward-facing gaze.

2.4 Procedure

Interested participants scheduled an appointment at their convenience. Following consent, individuals were assigned to one of three groups: 500-ms CS-US paired, 1000-ms CS-US paired or 1000-ms CS-US explicitly unpaired. Participants filled out the AMBI/RMBI and State/Trait prior to eyeblink classical conditioning. Participants were then fitted with EMG electrodes, the signal quality was checked, and conditioning began. Each participant received three US alone stimuli to establish appropriate responses to the airpuff and measure the UR prior to conditioning. Participants in paired conditioning were exposed to 60 CS-US paired trials with 500-ms or 1000-ms CS duration co-terminating with a 50-ms airpuff US. Those placed in unpaired conditioning received 60 CS and 60 US explicitly unpaired presented in pseudo random order (with no more than three consecutive occurrences of either stimulus in a row). Trials were separated by an inter-trial interval ranging from 25-37 s ($M=30$).

2.5 Signal Processing

Eyelid EMG recordings were evaluated for each participant on a trial-by-trial basis. To be recorded as an eyeblink response the smoothed signal must change by more than the mean activity plus 4 times the standard deviation in a 125-ms
comparator window [90]. Eyeblink sessions with excessive signal noise (loss of more than 10% of trials), incomplete session data (e.g., falling asleep), or that demonstrated a lack of a UR were discarded and not used from further analysis. Inspection of eyeblink conditioning sessions resulted in rejection of data from 45 participants, similar rejection rates were seen in previous human eyeblink studies [28, 29, 76, 100].

3. Results

3.1 Psychometric

There were no significant differences of survey scores as a function of group assignment (all p’s > .102) nor was the distribution of male and female participants different between groups, $\chi^2(1, N=118) = .542, p = .461$. Given previous research with these measures in eyeblink conditioning [100], we chose to concentrate primarily on AMBI scores to establish comparison groups. Participants were classified into inhibited and non-inhibited groups by using the median of the sample (15.5) for each measure. Mean scores and demographic information for the three conditions are listed in Table 1.

3.2 Eyeblink

We used a step-wise approach in this analysis. First, to see if there are any differences of acquisition in the paired CS-US learning condition we used a 2 (Group: High/Low AMBI) x 2 (Condition: 500/1000) x 6 (Acquisition Block) mixed measures ANOVA which revealed a Group x Acquisition Block interaction, $F(5,480) = 7.106, p < .01$, indicating that the 500-ms condition acquired the conditioned response but 1000-ms paired condition did not, see Figure 1. Of greatest interest is the three-way interaction of Group x Condition x Acquisition Block, $F(5,480) = 2.352, p = .04$. Visual inspection of the curves indicate that at 500-ms the high AMBI group learned
significantly faster than the low AMBI group, but these learning differences are not present in the paired 1000-ms condition, see Figure 2. There was no difference of initial UR amplitudes between the conditions, $F(2,112)=1.271, p=.284$, or between the AMBI groups, $F(1,112)=.216, p=.643$. Analysis of the other self-report measures used (RMBI, State, and Trait) revealed no significant learning differences, all $p$’s > 0.36. Finally, no significant sex differences were observed in acquisition, $F(5,480) = .580, p = .715$.

Longer CS durations are prone to capture a greater number of non-specific blinks simply because the window in which a CR can be produced is larger. To assess the prevalence of non-specific blinks at 1000-ms we compared the CS-US paired condition to an explicitly unpaired condition that presented the CS and US stimuli separately, allowing analysis of blinks to the presentation of the tone alone without the paired airpuff. A $2 \times 2 \times 6$ mixed measures ANOVA revealed a main effect of condition, $F(1,68) = 16.012, p< .001$, with a greater percent CR in the 1000-ms paired condition, see Figure 3.

4. Discussion

Considering the transition to clinical anxiety during adolescence and early adulthood, it follows that understanding anxiety vulnerability at this key period is essential. Here, we extend previous research in adults to demonstrate that that eyeblink conditioning is also useful in differentiating vulnerability to anxiety disorders in adolescents. Further, acquisition of the conditioned eyeblink response was assessed under different CS durations.

Overall, we found that learning was significantly better at the 500-ms CS duration. At this duration, learning was not much different than has been previously
reported in samples of college-aged adults [29, 76, 100]. We found that learning was significantly reduced at the 1000-ms paired CS duration, similar to what is observed in Herbert et al. [104] in adult long delay with a CS duration of 1250-ms. These findings are consistent with previous research using infant rats demonstrating acquisition under long delay is more similar to trace than standard delay procedures [113], although studies have reported the opposite, suggesting similarities between long-delay and standard delay conditioning using young adult rabbits [114, 115]. It is clear that continued research using long-delay methods is necessary to fully understand the essential underlying neural substrates and behavioral outcomes in humans.

Previously, we demonstrated that those who score high on AMBI acquire standard delay eyeblink conditioning to a greater extent. This initial finding demonstrated an important relationship between self-report measures of anxiety vulnerability and associative learning, suggesting that there is something fundamentally different about how inhibited individuals process the stimuli in their environment. Here, we compare acquisition of standard delay to a longer CS duration. Differences in learning at these two CS durations shed light on underlying neural substrates influencing facilitated acquisition. We chose to concentrate on hippocampal involvement based on previous research indicating the hippocampus may be involved in risk for anxiety in terms of structure [116], function [117], and behavior [65].

Hippocampal dysfunction in inhibited individuals is one viable explanation for previously observed learning differences in standard eyeblink conditioning. Reduced hippocampal interference in the high AMBI group would result in faster learning at 500-ms and a deficit in learning at 1000-ms, when the hippocampus is essential to
successfully acquire the CR. Another explanation is that the cerebellum itself is dysfunctional. Recent neuroimaging studies indicate increased cerebellar activity in Lobule VI of inhibited individuals to novel faces [37], as well as during eyeblink conditioning [118]. It is possible that individual differences in activity in the Lobule VI region are contributing to facilitated eyeblink conditioning in high AMBI. Here, we found that high AMBI learned significantly faster at 500-ms, supporting previous research in college-age samples and extending these individual differences to adolescence. No differences in learning were observed at the 1000-ms paired condition, indicating that hippocampal dysfunction is not mediating differences observed at 500-ms – if this were the case we would expect to see poorer learning of the high AMBI compared to the low AMBI groups. The lack of learning differences at 1000-ms paired indicates that individual differences in cerebellar activity are more likely responsible for facilitated learning at 500-ms, however cerebellar involvement in the inhibited group was not enough to maintain learning in the more difficult long delay duration.

Future research in this area will contribute to understanding the role of the cerebellum in anxiety vulnerability. In recent years interest has increased in the higher cognitive capacities of the cerebellum (for a review see [119]). Tract-tracing studies using transneuronal retrograde viruses indicate reciprocal connections between the non-motor cortical regions such as the dorsolateral prefrontal cortex and the cerebellar dentate nucleus [120, 121]. Using resting state functional connectivity, researchers have demonstrated connections between non-motor cortical regions and distinct areas of the cerebellum, largely Lobule VI, Crus 1 and Crus 2 [51, 52, 122]. Structural and functional findings are supported by clinical studies reporting that not all cerebellar insults result in motor deficits. In fact, some individuals have minor if
any motor impairments and instead suffer from behavioral changes affecting executive functioning, verbal fluency, working memory, abstract reasoning, personality and language [123, 124]. Finally, task-based neuroimaging studies have reported increased cerebellar activity in emotional, language, memory and executive tasks [125].

4.1 Conclusions

Prior studies make a clear case that the cerebellum is involved in higher cognitive functions, including those related to emotion and executive functioning. Here, we demonstrate behaviorally inhibited individuals show greater learning in a 500-ms eyeblink conditioning paradigm but not to 1000-ms, suggesting a cerebellar role underlying learning differences in behavioral inhibition. Cerebellar dysfunction may affect reciprocal cerebro-cerebellar pathways and influence regions involved in stimulus processing and avoidance, resulting in individual differences in behavioral inhibition. Continued research of the cerebellar contribution to individual differences observed in behaviorally inhibited temperament will shed important light on the role of the cerebellum in anxiety vulnerability.
Table 1. Number of participants and mean score on the Adult Measure of Behavioural Inhibition in each condition.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Inhibited High AMBI (&gt;15.5)</th>
<th>Non-Inhibited Low AMBI (&lt;15.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean AMBI (SD)</td>
</tr>
<tr>
<td>500-ms Paired</td>
<td>21</td>
<td>19.62 (3.56)</td>
</tr>
<tr>
<td>1000-ms Paired</td>
<td>26</td>
<td>20.42 (3.52)</td>
</tr>
<tr>
<td>1000-ms Unpaired</td>
<td>11</td>
<td>19.9 (3.80)</td>
</tr>
</tbody>
</table>

AMBI = Adult Measure of Behavioural Inhibition. SD = Standard Deviation
Figure 1. Acquisition of the eyeblink CR for groups receiving a 500-ms or 1000-ms paired CS duration. The acquisition session consisted of 6 blocks of 10 paired CS/US trials. Significant acquisition of the CR is apparent for the 500-ms CS duration, but not the 1000-ms paired CS duration as evidenced by a Group x Block interaction. Error bars represent the standard error of the mean.
Figure 2. Acquisition of the eyeblink CR for 500-ms (left panel) and 1000-ms paired (right panel) as a function of AMBI group. AMBI was assessed via the Adult Measure of Behavioural Inhibition (Gladstone & Parker, 2006) with those scoring above the median classified as High AMBI and those scoring below the median as Low AMBI. There was a significant interaction of Group x Block x Condition, with faster CR acquisition for the High AMBI group in the 500-ms CS duration condition, a difference not evident in the 1000-ms paired condition. Error bars represent the standard error of the mean.
Figure 3. Acquisition of the eyeblink CR for 1000-ms explicitly unpaired compared to paired eyeblink conditioning. A Group x Block x Condition mixed measures ANOVA revealed a main effect of condition. Error bars represent the standard error of the mean.
Study 3

Individual differences in resting-state functional connectivity with the executive network: Support for a cerebellar role in anxiety vulnerability

Caulfield, M.D.\textsuperscript{1,2,3}, Zhu, D.C.\textsuperscript{4,5}, McAuley, J.D.\textsuperscript{2,5}, Servatius, R.J.\textsuperscript{1,2,3}

\textsuperscript{1}Graduate School of Biomedical Sciences, Rutgers University, Newark, NJ, USA; \textsuperscript{2}Stress and Motivated Behavior Institute, East Orange, NJ, USA; \textsuperscript{3}Department of Veterans Affairs, New Jersey Health Care System, East Orange, NJ, USA; \textsuperscript{4}Department of Radiology, Michigan State University, East Lansing, MI, USA; \textsuperscript{5}Department of Psychology, Michigan State University, East Lansing, MI, USA.

Under Review, \textit{Brain Structure and Function}
Abstract
This study characterized cerebellar connectivity with executive intrinsic connectivity networks. Using the right and left dorsolateral prefrontal cortices (dIPFC) and right orbital frontoinsula as seed regions, we measured resting-state brain connectivity in healthy college-aged participants. Based on previous research demonstrating a relationship between the cerebellum and self-report measures of behavioral inhibition, we assessed individual differences in connectivity between groups. Overall, intrinsic activity in cerebellar lobule VIII was significantly correlated with the executive network and cerebellar Crus I with the salience network. Between-group comparisons indicated cerebellar connectivity with the executive network in behaviorally inhibited individuals. Intrinsic activity in Crus I, a region previously implicated in non-motor cerebellar functions, significantly correlated with intrinsic activity in the right dIPFC seed region. These findings support a growing number of studies demonstrating cerebellar influence on higher cognitive processes, extending this relationship to individual differences in anxiety vulnerability.
1. Introduction

The cerebellum is traditionally thought of as a motor structure. However, awareness that the cerebellum plays a role in higher cognitive functions is growing. Evidence from anatomical studies in primates [40, 120] and clinical work in humans [123, 126-129] supports a growing number of imaging studies reporting cerebellar activity that is not linked to motor behavior, such as emotion and attention [130-136].

The cerebellum has widespread connections to both motor regions of the primary and premotor cortex and non-motor regions including prefrontal areas [40, 137, 138]. Detailed anatomical studies indicate a discrete topography organizing cerebellar input and output to cortical areas [139, 140]. Using tract-tracing methods in primates, Strick and colleagues demonstrate that non-motor regions of the prefrontal cortex (Brodmann areas 9, 46, and 12) have distinct connections to the dentate nucleus of the cerebellum [120, 139]. Critically, they demonstrated that the lateral dentate nucleus projects to the prefrontal cortex (PFC), with separate dorsal dentate projections terminating in the motor and premotor regions, indicating a topographic organization of the dentate nucleus supporting both motor and non-motor output to the cortex [139]. These results have led to the proposal that the cortico-cerebellar connections are via reciprocal “parallel circuits” that connect the cerebellum to the cortical regions, and vice versa [121].

Cerebellar connectivity to non-motor areas is also supported by recent research using resting-state functional magnetic resonance (fMRI) imaging techniques. Resting-state fMRI is based on the theory that fluctuating brain activity at rest correlates between brain regions, reflecting intrinsic functional networks of the brain [42, 141]. For example, regions typically associated with motor function tend to activate in synchrony, waxing and waning together, and are therefore considered to
form a particular network [44, 45]. Studies have demonstrated intrinsic connectivity networks (ICNs) corresponding to basic neural functions such as sensorimotor, vision, audition, language, executive function and salience detection [44-50, 142]. Recent work analyzing subcortical contributions to the ICNs has paid little attention to cerebellar connectivity. Those studies that have assessed cerebellar connectivity with the ICNs have been promising, demonstrating cerebellar contributions to all functional networks [51]. Importantly, these studies have shown that regions of the cerebellum contribute distinctly to individual networks, with some regions such as cerebellar Crus I, Crus II and lobule VI contributing specifically to cortical networks such as the executive control and salience networks [51, 52, 122].

Resting-state fMRI provides a useful platform to study individual differences in connectivity in both clinical and non-clinical populations. So far, anxiety researchers have primarily considered the possibility of intrinsic connectivity differences in psychopathology, such as individuals with social anxiety disorder [55-57, 143], obsessive compulsive disorder [58] and post-traumatic stress disorder (PTSD; [59-62, 144, 145]. However, individual differences in intrinsic connectivity may be a pre-existing risk factor for the development of an anxiety disorder. For example, a stress-diathesis approach suggests that anxiety is due to the interaction of risk factors such as sex, genetics, personality [1], brain structure [64] and learning [76, 100]. It is likely that connectivity may also play an essential role in mediating risk for developing clinical anxiety.

Recent research supports a diathesis approach to understanding anxiety vulnerability. Individual differences in learning performance were found on a cerebellar-mediated associative learning task [100]. In this task, participants were given a battery of measures related to risk for anxiety including the Adult Measure of
Behavioural Inhibition (AMBI) [20], a measure of behavioral inhibition that has been linked to greater risk for developing clinical anxiety [5-7, 146], and then underwent eyeblink classical conditioning. Those with high scores on the AMBI learned significantly faster and to a greater degree than those with low scores, suggesting that there is something fundamentally different about how those at risk for an anxiety disorder learn about their environments. However, aside from a handful of electroencephalography (EEG) studies examining resting-state EEG [147, 148] no studies to date have assessed individual differences in intrinsic connectivity of behaviorally inhibited individuals using resting-state fMRI.

It remains unclear why those with behaviorally inhibited temperament (BI) demonstrate individual differences in eyeblink conditioning [76, 100], as well as enhanced avoidance learning [65]. Reciprocal connectivity between the cerebellum and cortical regions places it in a position to modulate higher cognitive processes via connections with the dorsolateral prefrontal cortex (dIPFC). In addition to its role as a major structure in the executive ICN [45], the dIPFC also plays an important role in many executive functions including approach and avoidance motivation [149, 150].

The present study had two purposes. The first was to further delineate the role of the cerebellum in specific non-motor ICNs. The second was to assess individual differences in connectivity with intrinsic connectivity networks implicated in anxiety vulnerability. Given the behavioral profile observed in BI, the executive network and salience network are the most likely candidates to demonstrate individual differences. As such, recent research studies have reported increased resting-state connectivity of the amygdala and insula in Veterans with PTSD, implicating the salience network [62]. However, given previous research in cerebellar-mediated eyeblink conditioning [76, 100] avoidance learning [65] and task-
based fMRI [37], we hypothesized that there would be regions in the cerebellum that will be significantly correlating with executive network in resting-state fluctuations, with inhibited individuals demonstrating greater cerebellar connectivity with the executive network.

2. Methods

2.1 Participants

Twenty-six young adults (n=19 female, n=7 male), ages 18-25 (M=20.7, SD=1.8), from a large midwestern university participated in the study. All study materials were reviewed and approved by internal review and informed consent was obtained from all participants prior to any experimental procedures.

2.2 Psychometric Scales

Participants completed a battery of self-report measures including the Adult and Retrospective Measures of Behavioral Inhibition [20] and the Spielberger State/Trait Anxiety Inventory [17]. Participants were part of a larger study on behavioral inhibition and as such were classified as behaviorally inhibited if their AMBI score was above the median of 11 and non-inhibited if they scored below the median [100]. Except for AMBI score, there were no significant differences between group demographics, all t's > .603, see Table 1.

The Adult Measure of Behavioral Inhibition is a 16-item self-report measure that assesses inhibition or avoidance in response to new stimuli or social situations. Scores range from 0-32 and include questions such as “Do you tend to withdraw and retreat from those around you?” and “Do you tend to introduce yourself to new people?” [20]. The Spielberger State/Trait Anxiety Inventory (STAI) is a 40-item self-report questionnaire with total scores ranging from 40 to 160. The STAI is separated into two parts, State and Trait anxiety, each consisting of 20 questions: State Anxiety
is assumed to change with mood and emotion and asks questions about the current emotional state of the participant such as “I am tense” and “I feel at ease”. Trait Anxiety is a relatively stable personality characteristic and asks questions about general feelings and behaviors such as “I feel nervous and restless” and “I feel satisfied with myself” [17].

2.3 Imaging data acquisition

The MRI experiment was conducted on a GE 3T Signa® HDx MR scanner (GE Healthcare, Waukesha, WI) with an 8-channel head coil. During each session, first and higher-order shimming procedures were carried out to improve magnetic field homogeneity. To study resting-state brain function, a 7-min echo-planar imaging datasets, starting from the most inferior regions of the cerebellum, were acquired with the following parameters: 44 contiguous 3-mm axial slices in an interleaved order, time of echo (TE) = 20 ms, time of repetition (TR) = 2500 ms, parallel acceleration factor = 2, flip angle = 80°, field of view (FOV) = 22 cm, matrix size = 64 × 64, ramp sampling, and with the first four data points discarded. Each volume of slices was acquired 164 times while a subject was asked to relax, stay awake and keep his eyes closed. After the functional data acquisition, 180 T₁-weighted 1-mm³ isotropic volumetric inversion recovery fast spoiled gradient-recalled images (10 minute scan time), with cerebrospinal fluid (CSF) suppressed, were obtained to cover the whole brain with the following parameters: TE = 3.8 ms, TR of acquisition = 8.6 ms, time of inversion (TI) = 831 ms, TR of inversion = 2332 ms, flip angle = 8°, FOV = 25.6 cm × 25.6 cm, matrix size = 256 × 256, slice thickness = 1 mm, and receiver bandwidth = ± 20.8 kHz.

3. Resting-State fMRI Individual-Subject Data Pre-Processing
Resting-state fMRI correlation analysis was conducted using AFNI software [151] in the native space. For each subject, the acquisition timing difference was first corrected for different slice locations. With the last functional volume as the reference, rigid-body motion correction was done in three translational and three rotational directions. The amount of motion was estimated and then modeled in data analysis. For each subject, spatial blurring with a full width half maximum (FWHM) of 4 mm was used to reduce random noise and inter-subject anatomical variation during group analysis. At each voxel, motion-estimation parameters, baseline, linear and quadratic system-induced signal trends were removed from the time courses using the “3dDeconvolve” routine in AFNI [152]. Brain global, CSF and white matter (WM) mean signals were modeled as nuisance variables and removed from the time courses as well. In order to create the time course from pure CSF regions, the lateral and 3rd ventricles on the high-resolution T1-weighted volumetric images were segmented using FreeSurfer software followed by 1 mm³ erosion [153]. For the same reason, the WM was segmented from the T1-weighted volumetric images using the “FAST” routine in the FSL software [154] followed by 4 mm × 4 mm × 4 mm cubical erosion. The cleaned time courses were then band-pass filtered in the range of 0.009 Hz – 0.08 Hz [142]. These filtered time courses were used for correlation-based connectivity analyses following.

3.1 Generation of Seed Regions

Previous research indicates that individuals with behavioral inhibition may react differently to novel situations, people, or stimuli [37, 117]. Additionally, the dorsolateral prefrontal cortical region has been indicated as important in processing novelty in the environment [155] as well as in avoidant behaviors [149, 150, 156]. Given the relationship between behavioral inhibition, attention to novelty, and
avoidance we chose to place seed regions in the right and left dlPFC, a central structure in the executive network. Spherical seed regions with radii of 6 mm were placed for each participant in the right dlPFC (x=42,y=41,z=23) and left dlPFC (x=-45, y=44, z=20) to assess connectivity with the executive network. Additionally, seed regions with 6 mm radii were placed for each participant in the right orbital frontoinsula (x=38, y=12, z=-8) to assess connectivity with the salience network [45]. Each seed was visually assessed against anatomy and the location was modified if necessary.

3.2 Functional Connectivity between executive network seeds and Rest of the Brain

The “3dfim+” routine in AFNI [151] was used to correlate the time course in every voxel of the brain against the space-averaged time course from a seed region. To prepare for group analysis, the correlation coefficients were converted to Z values through Fisher’s Z-transformation to improve the normality of the distribution. The Z values were then warped to the MNI 305 standard space through the FreeSurfer non-linear registration pipeline. After warping to the MNI305 standard space, the data were spatially blurred with FWHM of 2 mm to reduce potential noise generated by non-linear warping. Between-group t tests were performed, as well as the whole group vs. baseline.

Monte Carlo simulation was performed according to the matrix and voxel size of the imaging volume, voxel intensity thresholding, masking and spatial smoothness of image data inherited and applied. The spatial smoothness of image data was estimated based on “3dFWHMx” in AFNI [151]. The cluster analysis was used to estimate the overall statistical significance with respect to the whole brain [152]. The between-group t-test results for functional connectivity with the seed region were corrected for multiple comparisons based on the following criteria: A voxel was
considered significant only if it was within a cluster of at least 1170 voxels in which the voxels were connected and all had a voxel-based \( p \leq 0.005 \). Based on the application of these criteria to the whole brain, the voxel-based \( p \leq 0.005 \) was corrected to be an equivalent whole-brain corrected \( p \leq 0.047 \).

4. Results

4.1 Whole group analysis

The results of functional connectivity analyses over all participants are presented in Table 2. The right and left dlPFC seed regions showed significantly greater connectivity with a number of bilateral regions related to executive functioning as well as cerebellum lobule VIII, see figure 1. The right orbital frontoinsula had significantly greater connectivity with bilateral Brodmann’s area 40 as well as the left cerebellum Crus I, see figure 2.

4.2 Between-group analyses

High and Low AMBI groups were compared using an independent samples \( t \)-test to ensure significant group differences in self-report scores, \( t(24) = -8.922, p<.001 \). A contrast of regions of differentially correlating activity with the dlPFC between the high and low AMBI groups revealed significantly greater connectivity of the high AMBI group between the left dlPFC seed and the dorsal anterior cingulate cortex, which has been linked to attention, emotion and avoidance [149, 157, 158], see Table 3. For the right dlPFC seed, the high AMBI group demonstrated significantly greater connectivity with the cerebellum Crus I, see Figure 3. Between group comparisons for the orbital frontoinsula seed revealed no significant differences.

5. Discussion
The cerebellum continues to gain acceptance for its role in cognitive processes. In this study, resting-state fMRI was used to demonstrate intrinsic cerebellar connectivity with non-motor executive connectivity networks and to assess how connectivity of the cerebellum may differ as a function of anxiety vulnerability. Previous research using retroviral tracing techniques have demonstrated that the Crus I and Crus II of the cerebellum have distinct reciprocal connections to non-motor prefrontal cortical regions [121]. These findings support recently published studies establishing connectivity of the Crus I and Crus II with the executive network and other prefrontal cortical regions [51, 122]. The present findings provide further support for a cerebellar role in the executive and salience networks.

Overall, we also observed correlations of the intrinsic activity of the cerebellum Crus I and orbital frontoinsula, supporting the conclusion that the cerebellum plays a role in non-motor functioning. The specific correlation of Crus I with the orbital frontoinsula supports distinct contributions previously demonstrated by Habas [51]. For the executive network, intrinsic activity in the cerebellum lobule VIII was correlated with intrinsic activity in the dlPFC. It is less clear, however, what role(s) lobule VIII plays in the executive functions of the cerebellum. Clinical studies report strokes in lobule VIII can alter the subjective experience of pleasant feelings [159], suggesting it may be involved in emotional processing. A recent meta-analysis reports that cerebellar lobule VIII activated in emotional, language, music and working memory tasks, often co-activating with Crus I and Crus II [125], similar to what we observed with the left dlPFC seed region. As research on the cognitive functions of the cerebellum moves forward more light may be shed on the relationship between lobule VIII and the dorsolateral prefrontal cortex.
An essential component of this study was the between-group comparison between individuals at greater and lesser risk for the development of an anxiety disorder. Previous research in a cerebellar-dependent associative learning task [100] implies that the cerebellum may play an important role in behavioral inhibition and anxiety vulnerability. We compared behaviorally inhibited (high AMBI) to non-inhibited (low AMBI) participants, using seed regions placed individually for each subject to assess potential differences in functional connectivity between the two groups. Results revealed that intrinsic connectivity between the cerebellum and executive network is influenced by individual differences in anxiety vulnerability as measured by AMBI scores. These differences were not observed between groups in the salience network. Critically, behaviorally inhibited individuals show significantly greater connectivity between the right dlPFC and the right cerebellum Crus I. This finding is supported by previous studies demonstrating that extensive portions of the Crus I/Crus II map to prefrontal cortical regions involved in executive control [51, 52]. Here, we have demonstrated that previously reported connectivity may be driven by individual differences.

Research has increased in executive network dysfunction in recent years in areas such as Alzheimer’s and schizophrenia (for review see: [160] but has not assessed anxiety vulnerability. Even more specifically, the role of the cerebellum in anxiety vulnerability has been largely overlooked. The dlPFC, has been shown to be both an essential component of the executive connectivity network [45], and important in processing novelty in the environment and avoidance behaviors [150, 155, 156] – both of which are representative of the behavioral profile seen in behavioral inhibition. Here, we report that the cerebellum is differentially active in those with behavioral inhibition, suggesting that cerebellar connectivity may play an
important role in mediating anxiety. Since this research is at a young stage it is difficult to determine exactly what role the cerebellum is playing, although multiple possibilities exist. One is that the increased reliance on the cerebellar circuitry may be in response to dysfunction in other systems. For example, hippocampal differences have been shown to be related to the development of PTSD [116].

Furthermore, behaviorally inhibited individuals show enhanced avoidance in a hippocampal dependent avoidance task [65]. It is possible that increased reliance on cerebro-cerebellar circuitry may be the result of hippocampal dysfunction. Another possibility is that cerebro-cerebellar circuitry itself is dysfunctional resulting in individual differences observed in cerebellar-dependent learning tasks [100] and in connectivity between the cerebellum and cognitive regions within its circuitry.

Behaviorally inhibited individuals showed significantly greater connectivity between the left dIPFC and right dorsal anterior cingulate (dACC). Increased connectivity of high AMBI between the dACC and dIPFC may play an important role in anxiety vulnerability as it is associated with error detection, anticipation, attention, emotional responses and avoidance [149, 157, 158]. Although there were no significant cerebellar differences with the left dIPFC seed, these findings are relevant in terms of the profile of behavior observed in behavioral inhibition, especially in respect to attention, avoidance, and emotion. These findings suggest that those with behaviorally inhibited temperament may have greater connectivity between the executive network and areas responsible for attention, avoidance, anticipation and error detection. Such increased connectivity may result in differences in processing of stimuli in the environment. For example, if the regions responsible for vigilance are connected with those responsible for attention, error detection, and avoidance, then the system may misinterpret non-threatening circumstances for situations that call for
vigilance. Increased vigilance may lead to internal and external feelings of anxiety and contribute to the cycle of avoidance that is an essential component in the development of anxiety.

As this is only the first study of its kind it is clear that continued research is necessary to fully understand individual differences in functional connectivity and how it relates to anxiety vulnerability. Presently, explanations for anxiety vulnerability concentrate on prefrontal and amygdalar structures, overlooking subcortical regions such as the cerebellum. A full understanding of the relationship between these regions may shed light on the neural substrates underlying anxiety vulnerability.
Table 1. Group mean demographic details.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Females (n)</th>
<th>AMBI (SD)</th>
<th>Age (SD)</th>
<th>Education (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High (scores &gt; 11.5)</td>
<td>13</td>
<td>10</td>
<td>16.3 (2.4)</td>
<td>20.8 (2.0)</td>
<td>14.8 (2.1)</td>
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<tr>
<td>Low (scores &lt; 11.5)</td>
<td>13</td>
<td>9</td>
<td>8.5 (2.1)</td>
<td>20.5 (1.7)</td>
<td>14.5 (1.4)</td>
</tr>
</tbody>
</table>

AMBI=Adult Measure of Behavioural Inhibition
Table 2. Significantly connected regions with the dorsolateral prefrontal cortex.

<table>
<thead>
<tr>
<th>R/L</th>
<th>Brain region</th>
<th>Max $t$ coordinate (x,y,z)</th>
<th>Cluster size (mm$^3$)</th>
<th>Max $t$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left dlPFC seed region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>Dorsolateral prefrontal cortex (BA 46/10)</td>
<td>-40, 37, 22</td>
<td>145290</td>
<td>19.3530</td>
</tr>
<tr>
<td>L</td>
<td>Inferior parietal lobule (BA 40)</td>
<td>-53, -54, 49</td>
<td>16386</td>
<td>7.3034</td>
</tr>
<tr>
<td>L</td>
<td>Middle temporal gyrus (BA 20)</td>
<td>-57, -48, -14</td>
<td>8083</td>
<td>6.3015</td>
</tr>
<tr>
<td>R</td>
<td>Inferior parietal lobule (BA 40)</td>
<td>44, -57, 49</td>
<td>6151</td>
<td>6.6122</td>
</tr>
<tr>
<td>R</td>
<td>Cerebellum VIII/Crus I/Crus II</td>
<td>26, -70, -66</td>
<td>5686</td>
<td>6.5349</td>
</tr>
<tr>
<td>R</td>
<td>Middle temporal gyrus (BA 20)</td>
<td>58, -50, -15</td>
<td>3928</td>
<td>6.6377</td>
</tr>
<tr>
<td>R</td>
<td>Extrastriate Cortex (18/19)</td>
<td>12, -76, -43</td>
<td>1626</td>
<td>6.2026</td>
</tr>
<tr>
<td>Right dlPFC seed region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>Dorsolateral prefrontal cortex (BA 46/10)</td>
<td>38, 45, 25</td>
<td>200981</td>
<td>18.8000</td>
</tr>
<tr>
<td>R</td>
<td>Inferior parietal lobule (BA 40)</td>
<td>52, -49, 49</td>
<td>22102</td>
<td>11.0190</td>
</tr>
<tr>
<td>L</td>
<td>Inferior parietal lobule (BA 40)</td>
<td>-46, -58, 46</td>
<td>7767</td>
<td>6.2433</td>
</tr>
<tr>
<td>R</td>
<td>Middle temporal gyrus (BA 37)</td>
<td>54, -52, -9</td>
<td>7351</td>
<td>6.0750</td>
</tr>
<tr>
<td>L</td>
<td>Cerebellum VIII</td>
<td>-24, -68, -69</td>
<td>3897</td>
<td>5.4811</td>
</tr>
<tr>
<td>R</td>
<td>Precuneus (BA 7/5)</td>
<td>5, -41, 49</td>
<td>1221</td>
<td>5.1153</td>
</tr>
<tr>
<td>Right orbital frontoinsula seed region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>Insula</td>
<td>40, 7, -6</td>
<td>182692</td>
<td>16.4300</td>
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<tr>
<td>R</td>
<td>Inferior parietal lobule (BA 40)</td>
<td>58, -45, 38</td>
<td>10108</td>
<td>7.4944</td>
</tr>
<tr>
<td>L</td>
<td>Inferior parietal lobule (BA 40)</td>
<td>-66, -31, 24</td>
<td>4991</td>
<td>6.1427</td>
</tr>
<tr>
<td>L</td>
<td>Cerebellum Crus I</td>
<td>-40, -49, -41</td>
<td>2581</td>
<td>5.5327</td>
</tr>
</tbody>
</table>

BA=Brodman’s Area
Table 3. Regions of greater connectivity for high AMBI compared to low AMBI

<table>
<thead>
<tr>
<th>R/L</th>
<th>Brain region</th>
<th>Max t coordinate (x,y,z)</th>
<th>Cluster size (mm$^3$)</th>
<th>Max t value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left dlPFC seed region</td>
<td>Dorsal Anterior Cingulate Cortex</td>
<td>6, 16, 47</td>
<td>1186</td>
<td>3.9803</td>
</tr>
<tr>
<td>Right dlPFC seed region</td>
<td>Cerebellum Crus I</td>
<td>34, -73, -36</td>
<td>1844</td>
<td>5.0489</td>
</tr>
</tbody>
</table>

BA=Brodmann’s Area dlPFC=dorsolateral prefrontal cortex
Figure 1. Matching slices (x= -24, y= -68, z= -69) demonstrate similar connectivity between the cerebellum and executive network seeds. Intrinsic activity of the left dorsolateral prefrontal cortex was significantly correlated with activity in the right cerebellum lobule VIII (left panel) and the right dorsolateral prefrontal cortex with left lobule VIII (right panel).
Figure 2. The cerebellum Crus I (x= -40, y= -49 z= -41 shown here) also showed significant correlations of intrinsic activity with the orbitofrontal insula seed of the salience network.
Figure 3. Between-group comparisons indicated significantly greater cerebellar connectivity of the Crus I (34, -73, -36) with the executive network in behaviorally inhibited individuals.
Study 4

Differential activation to familiar and novel stimuli in those vulnerable to anxiety disorders.

Caulfield, M.D.¹²³, Zhu D.C.⁴⁵, McAuley, J.D.²⁵, Servatius, R.J.¹²³

¹Graduate School of Biomedical Sciences, Rutgers University, Newark, NJ, USA; ²Stress and Motivated Behavior Institute, East Orange, NJ, USA; ³Department of Veterans Affairs, New Jersey Health Care System, East Orange, NJ, USA; ⁴Department of Radiology, Michigan State University, East Lansing, MI, USA; ⁵Department of Psychology, Michigan State University, East Lansing, MI, USA.
Abstract
This study assessed individual differences in reactivity to familiar and novel stimuli. Using a cerebellar-centered approach, we measured reactivity to familiar and novel faces and scenes in healthy college-aged participants. Participants were given a self-report measure of behavioral inhibition, a temperament associated with avoidance of novel people or situations. Activity in cerebellar lobule IV-IX was significantly greater for familiar faces compared to novel faces in behaviorally inhibited individuals. Comparisons of reactivity to scene stimuli revealed significantly greater activity to novel scenes than familiar scenes in the dorsolateral prefrontal cortex. Overall, these findings suggest that the cerebellum is active during non-motor processing tasks. Additionally, individual differences in reactivity of the cerebellum and dorsolateral prefrontal cortex may indicate alterations in connectivity of the cerebro-cerebellar circuitry that may influence the development of anxiety disorders.
1. Introduction

The cerebellum is traditionally known for its essential involvement in motor behavior. However, a growing body of research is converging to suggest that the cerebellum also plays a role in higher cognitive functions. Reciprocal pathways between the cerebellum and cortex via afferent connections of the cortico-pontine-cerebellar tract are already in place to carry motor demands to the cerebellum [161-163]. Recent advances in tract-tracing methods demonstrate reciprocal connectivity between the cerebellum and the dorsolateral prefrontal cortex of the primate [121]. It is via these parallel loops that the cerebellum may modulate regions responsible for higher cognitive functions including behavioral control, working memory, decision marking, reward and expectancy, and emotion and motivation [164-168].

Cerebellar connectivity to non-motor cortical regions using neuroimaging reflects tract-tracing studies. Recent work analyzing cerebellar contributions to the intrinsic connectivity networks has been promising, demonstrating cerebellar contributions to all functional networks [51, 52, 122, 169]. Importantly, regions of the cerebellum contribute distinctly to individual networks, suggesting a specific topography of the cerebellum, with some regions exclusively connected to non-motor cortical areas. This is further supported by task-based imaging studies reporting increased cerebellar activity during cognitive functions such as executive, language, music, working memory and emotion (for review see: [125]).

The cerebellum is involved in a variety of pathologies including schizophrenia, attention deficit hyper-activity disorder, autism, fetal alcohol syndrome, and anxiety disorders [170-178]. While many reports indicate cerebellar activity in anxiety disorders, only a few of these studies specifically assessed the cerebellar role in anxiety, with most concentrating on the amygdala, hippocampus, or
prefrontal structures. Analysis of cerebellar stroke has revealed non-motor deficits affecting executive functioning, verbal fluency, working memory, spatial memory, personality, and language [123, 124]. The combined anatomical, connectivity, functional and clinical evidence suggests the cerebellum may play an essential role in anxiety vulnerability. Future research concentrating specifically on cerebellar involvement is warranted.

Little is understood about what makes an individual more likely to develop an anxiety disorder. Partly, this is because there is no single factor related to increased risk for anxiety. A stress-diathesis model emphasizes the changes in stress reactivity from the convergence of a variety of factors such as genetics, biology, sex, prior experience, and temperament [1]. Although current research focuses primarily on higher cortical areas such as the prefrontal cortex, hippocampus, and amygdala, there is growing evidence that the cerebellum may play an important role as well.

One way of measuring anxiety vulnerability is through self-reports of temperament. Behavioral inhibition (BI) is a temperament that has been linked to the development of anxiety disorders [5-7]. Behavioral inhibition and anxiety share similar physiological and behavioral characteristics including the tendency to withdraw or avoid from novel situations or people, suggesting that avoidance is an essential component in the development and maintenance of anxiety [13, 69, 71]. Recent research demonstrates enhanced avoidance learning by individuals with BI in both operant [65] and eyeblink classical conditioning paradigms [100], suggesting a link between the cerebellum, avoidance and BI.

Additional support comes from neuroimaging. Blackford and colleagues [37] report that behaviorally inhibited individuals have greater cerebellar reactivity to novel faces compared to familiar faces in the right cerebellum lobule VI, Crus I.
Recent research using resting-state functional connectivity demonstrates cerebellar connectivity of the right Crus I to the right executive networks in behaviorally inhibited individuals (Caulfield, under review). Taken together, there is strong evidence that the cerebellum not only plays a role in higher cognitive processes, but that it may be an important neural substrate in anxiety vulnerability.

Using functional MRI (fMRI) methods to further understand cerebellar reactivity to visual stimuli, the present study was designed to assess individual differences of behaviorally inhibited individuals in reactivity to familiar and novel faces (as previously assessed by Blackford) and familiar and novel scenes. This approach allowed us to assess the specific effects of social stimuli via faces as well as reactivity to more general stimuli using scenes. We hypothesized that while there is a social component to the observed increased in cerebellar activity, increased activity would occur to both the novel faces and novel scenes.

2. Methods

2.1 Participants

Thirty-six students from a large Midwestern State University participated. All consent forms and study materials were approved by internal review. A total of 26 participants (19 females and 7 males, mean age 20.65, range 18-25) were included in the data analysis, see table 1. Data from ten subjects were discarded due to incomplete coverage of the cerebellum or excessive motion.

2.2 Psychometric Scales

Participants completed the Adult Measure of Behavioural Inhibition (AMBI;[20]). Based on a larger study of behavioral inhibition participants were classified as behaviorally inhibited if they scored above the median of 11 on the AMBI and non-inhibited if they were lower than the median [100]. Except for AMBI
score, there were no significant differences between group demographics, all $t'$s > .603.

2.3 Stimuli

Stimuli were 96 full-color digitized photographs. Face stimuli were of neutral expression faces of male and female adults from the AR face database [179] and scene stimuli were color images taken from typical rooms in a home (e.g., living room, kitchen, office, etc.) that were used in a previous imaging study [180]. The fixation cross was a 1" x 1" cross in black placed in the center of a white background. For representative examples of these stimuli see figure 1.

2.4 Procedure

Familiarization to a subset of stimuli (24 faces, 24 scenes) took place outside of the scanner at least 1 and no more than 3 days prior to functional MRI scanning. Participants were shown faces and scenes on a computer monitor for 1s each in randomized blocks (e.g., randomly presented faces, then randomly presented scenes) controlled by E-Prime (Psychology Software Tools, Inc., Pittsburgh, PA). Each photo was shown once per block (48 images per block), and each block was repeated six times totaling six 1 s presentations of each image. Face and scene groups were counter-balanced between participants.

2.5 Imaging Procedure

In the scanner, instructions were presented on the screen informing participants that they would see images from the familiarization day and new images. They were given a response pad and instructed to use their thumb to indicate “old” and their forefinger to indicate “new” in response to each stimulus while it was presented. Instructions were self-paced and participants were asked to make each type of response before proceeding.
A rapid event-related design paradigm was controlled by a computer equipped with E-Prime (Psychology Software Tools, Inc., Pittsburgh, PA). The visual stimuli were presented on a 1024 x 768, 32-in LCD monitor. The experiment was divided into four functional runs each lasting 7 min. In each run, baseline conditions and stimulus trials (6 per each condition, 24 total stimuli per each run) were pseudorandomly arranged using the RSFgen program in AFNI software (Cox, 1996) for optimizing the calculation of the hemodynamic response function for each stimulus condition. Each stimuli was presented for 2.5 s, with fixation intervals varying between 0-27.5 s. Participants were instructed to use the keypad to make the “old”/“new” response during each stimulus presentation.

2.6 MRI Acquisition

The MRI experiment was conducted on a GE 3T Signa® HDx MR scanner (GE Healthcare, Waukesha, WI) with an 8-channel head coil. During each session, first and higher-order shimming procedures were carried out to improve magnetic field homogeneity. Four runs of 7-min echo-planar imaging datasets, starting from the most inferior regions of the cerebellum, were acquired with the following parameters: 44 contiguous 3-mm axial slices in an interleaved order, time of echo (TE) = 20 ms, time of repetition (TR) = 2500 ms, parallel acceleration factor = 2, flip angle = 80°, field of view (FOV) = 22 cm, matrix size = 64 × 64, ramp sampling, and with the first four data points discarded. After the functional data acquisition, 180 T₁-weighted 1-mm³ isotropic volumetric inversion recovery fast spoiled gradient-recalled images (10 minute scan time), with cerebrospinal fluid (CSF) suppressed, were obtained to cover the whole brain with the following parameters: TE = 3.8 ms, TR of acquisition = 8.6 ms, time of inversion (TI) = 831 ms, TR of inversion = 2332 ms, flip
angle = 8°, FOV = 25.6 cm × 25.6 cm, matrix size = 256 × 256, slice thickness = 1 mm, and receiver bandwidth = ± 20.8 kHz.

3. fMRI Data Preprocessing and Analysis

All fMRI data preprocessing and analysis was conducted with AFNI software [151]. For each participant, acquisition timing differences was first corrected for different slice locations. With the last functional image as the reference, rigid-body motion correction was done in three translational and three rotational directions. The amount of motion was estimated and then the estimates were used in data analysis. For each participant, spatial blurring with a full width half maximum (FWHM) of 4 mm was applied to reduce random noise and inter-subject anatomical variation during group analysis.

For each participant, the impulse response function (IRF) was resolved with multiple linear regressions at each voxel with respect to each stimulus condition (including four conditions with correct trials and four conditions with incorrect trials) using the 3dDeconvolve routine in AFNI [152]. The IRFs were resolved to 7 points from 0 to 15 s at the resolution of 2.5 s (time of repetition). The BOLD signal change was calculated on the basis of the area under the IRF curve. General linear tests were applied on a voxel-wise basis for each of the stimulus conditions at the correct trials to inspect brain activation on individual subjects.

3.1 Whole-brain analysis

The cerebellum is a region of interest in this research. But the cerebellum is normally difficult to normalize to the standard space through a typical linear registration procedure. In this study, we applied the FreeSurfer non-linear registration pipeline [153]. This procedure was visually verified that the cortical and sub-cortical regions, as well as the cerebellum, could be normalized to the standard space with a
high level of accuracy, and has been successfully applied in Alzheimer’s research [53]. After the percent signal change was estimated for each participant at each stimulus condition, in the native space, the value was then warped to the MNI 305 standard space through the FreeSurfer non-linear registration pipeline. After warping to the MNI 305 standard space, the data were spatially blurred with FWHM of 2 mm to reduce potential noise generated by non-linear warping. A mixed-effects ANOVA was then performed over the entire (n=26) data set with stimulus condition (four types (all in correct trials): familiar face, novel faces, familiar scene, novel scene) as the fixed effect and participant as the random effect. ANOVA results were used to extract the differentially active voxels for all contrasts.

Monte Carlo simulation was performed according to the matrix and voxel size of the imaging volume, voxel intensity thresholding, masking and spatial smoothness of image data inherited and applied. The spatial smoothness of image data was estimated based on “3dFWHMx” in AFNI [151]. The cluster analysis was used to estimate the overall statistical significance with respect to the whole brain [152]. The statistical results were corrected for multiple comparisons based on the following criteria: A voxel was considered significant only if it was within an 1170 mm\(^3\) cluster in which the voxels were connected and all had a voxel-based \(p \leq 0.005\). Based on the application of these criteria to the whole brain, the voxel-based \(p \leq 0.005\) was corrected to be an equivalent whole-brain corrected \(p \leq 0.047\).

4. Results

4.1 Behavioral Results

Reaction times for responses to stimuli were measured in ms. A 2 (High AMBI/Low AMBI) x 2 (face_SCENE) x 2 (familiar/novel) mixed effects ANOVA revealed a significant interaction of face x familiar, \(F(1,24)=9.783, p= .005\). Overall,
participant’s reaction times to faces were faster than scenes, and familiar faces was fastest overall, see Table 2. There were no between group differences in reaction time to stimuli, all $p’s > .176$. Participants were able to recognize previously familiarized stimuli and correctly reject new stimuli. Using $d’$ as a measure of corrected recognition, a 2 (High AMBI/Low AMBI) x 2 (Face/Scene) mixed effects ANOVA revealed a significant interaction of AMBI group and accuracy, $F(1,24)= 10.181$, $p= .004$, with those scoring high on AMBI more accurate in the scenes condition, and those scoring low on AMBI more accurate in the faces condition, see Figure 2.

4.2 Imaging Results

First, to ensure that our stimuli were being processed as expected we extracted percent signal change from baseline for each stimulus type in regions of interest for processing faces, the fusiform face area (FFA [181]) and for processing scenes, the parahippocampal place area (PPA [180]). A 2 (stimulus: face/scene) x 2 (region: FFA/PPA) repeated measures ANOVA revealed a significant stimulus x region interaction, $F(1,25)= 5.322$, $p< .001$, such that percent signal change from baseline is greater at the FFA for faces and at the PPA for scenes.

Similar to Blackford et al. [37], we assessed activation of familiar compared to novel faces and scenes for the high AMBI and low AMBI groups separately, see table 3. In the high AMBI group, a large cluster of higher activity to the familiar than novel faces in the left cerebellum spanning lobules IV-IX and cingulate cortex that was not present in the low AMBI group, see Figure 3. Contrasts of familiar and novel scenes indicate higher activity of the dlPFC and caudate to novel than familiar scenes in the high AMBI group. The low AMBI group had greater activity to familiar scenes in the parahippocampal place area and precuneus. In addition to assessing
contrasts of each group separately, we performed between-group comparisons of the face and then scene contrasts, but no significant differences were found.

5. Discussion

This is the first study to assess the relationship between AMBI and reactivity to stimuli using fMRI. Inhibited individuals (high AMBI) demonstrated greater reactivity to familiar faces than familiar scenes in the cerebellum and cingulate cortex. These findings are unexpected given a previous neuroimaging study assessing individual differences in reactivity to novel and familiar faces [37]. The details of the current study and the Blackford study vary greatly, which may account for observed differences. One main difference is that the current study used the AMBI to classify participants as inhibited or non-inhibited. Blackford [37] used the Concurrent and Retrospective Self Report of Inhibition (CSRI/RSRI [18]) and selected only those participants with extreme scores on both measures. Our decision to use the AMBI was based on previous research demonstrating facilitated acquisition of classical eyeblink conditioning in those scoring high on AMBI, but not CSRI, suggesting that the AMBI may be a more sensitive measure of the construct of behavioral inhibition [100]. Along with these differences our adjustments to the procedure including a rapid-event related design and familiarization session outside of the scanner could have contributed to differences in the results between the studies.

Our decision to use a cerebellar-centered approach in imaging and to increase the scope of the study to scenes provides a new perspective on the cerebellar role in higher cognitive functioning. Although unexpected, we are confident in our findings as they are supported by replications within our study design of well-
established research observations such discriminant activity in the PPA and FFA and faster reaction times to familiar faces.

A noteworthy behavioral difference was observed between the groups. Those scoring high on AMBI were more accurate at identifying scenes, while those scoring low on AMBI were more accurate at identifying faces. Face recognition is one of the fastest and most accurate processes in humans [182]. Therefore, it is somewhat surprising that inhibited individuals show better recognition for scenes than faces in this study. Anxiety vulnerable and individuals with clinical anxiety display biased processing to stimuli in their environment, such that they demonstrate similar reactivity to all stimuli (positive, negative, neutral) as though it were negative or threatening [63, 183-187]. Although the face stimuli in the present study are categorized as neutral expression, it is possible that inhibited individuals interpret them as negative or threatening. This could alter the manner in which images are processed during familiarization, affecting subsequent recognition. The scenes, which lack the social aspect of faces, may be interpreted as less threatening and receive more processing by the inhibited individuals, improving their recognition memory. Future studies involving personally familiar faces or additional neutral stimuli may help to understand the observed recognition differences between groups.

Analysis of reactivity to stimuli in high and low AMBI indicated increased cerebellar activity to familiar faces. According to some theories, the cerebellum acts as a comparator, comparing demands from the cortex with reality to make fine adjustments. This process may be true not only for motor behavior but for attentional processes as well. If behaviorally inhibited participants operate on the expectation that all things in their environment will be novel and worthy of attention, then increased cerebellar activity may alter modulation of prefrontal processes, signaling
that the familiar faces are non-threatening. Another explanation is biased processing in behavioral inhibition. Regardless of how inhibited individuals classify the familiar faces, their initial reaction may be negative or threatening, increasing reactivity in regions that may be associated with novelty. It is likely that there may not be one specific region relating to anxiety vulnerability but dysfunction within the network. Continued understanding of the interplay between biased processing, expectation, and cerebro-cerebellar circuitry may prove useful in understanding individual differences in anxiety vulnerability.

The contrasts of the scene stimuli suggest that overall, high AMBI individuals are processing their environments differently than low AMBI. Two regions are significant for the low AMBI group, the parahippocampal gyrus and the precuneus, both of which are likely due to the scene stimuli and the old/new categorization task. Neither of these regions is significant in the high AMBI group. Rather, cortical structures responsible for behaviors observed in BI are once again involved. Increased activity of the dIPFC to novel scenes may be related to expectancy, emotion, behavioral control or decision making, which may be caused by the novel nature of the stimuli or response strategies used by participants. Regardless, the observed difference in the high AMBI group and not low AMBI group suggests that the dIPFC may play an essential role in behavioral inhibition.

Equally interesting is the increased caudate activity to novel scenes. As part of the basal ganglia, the caudate maintains reciprocal connectivity to the cerebral cortex – similar to the cerebellum. Additionally, the cerebellum is reciprocally connected with the basal ganglia, suggesting these structures may be essential subcortical influences on cortical processes [121]. The caudate is essential in learning and memory, but also may have cortical roles such as social behavior and
goal directed actions. As such, increased striatal activation has been reported in behavioral inhibition and social phobia [188].

Between-group analyses of the difference of familiar face vs. novel face and the difference of familiar scene vs. novel scene revealed no significant differences. Although we are seeing differences when activation is compared separately in high AMBI and low AMBI, our study has a lack of statistical power to capture such distinctions. Given that this is the first study of its kind to assess individual differences in AMBI, and that many of these findings fit with theories of behavioral inhibition and cerebellar contributions to higher cognitive processes, we feel that this study is an important contribution. Future research utilizing larger samples, or samples gathered from the tails of the distribution may reveal between-group differences. Additionally, the specific effects of faces and scenes in this study make interpretation difficult at times. Faces and scenes involve very specific neural processes that may alter activation patterns. Future studies using less specific stimulus types may reveal important individual differences in terms of familiar and novel stimuli in behavioral inhibition.
Table 1. Group mean demographic details.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Females (n)</th>
<th>AMBI (SD)</th>
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<th>Education (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High AMBI (scores &gt; 11.5)</td>
<td>13</td>
<td>10</td>
<td>16.3 (2.4)</td>
<td>20.8 (2.0)</td>
<td>14.8 (2.1)</td>
</tr>
<tr>
<td>Low AMBI (scores &lt; 11.5)</td>
<td>13</td>
<td>9</td>
<td>8.5 (2.1)</td>
<td>20.5 (1.7)</td>
<td>14.5 (1.4)</td>
</tr>
</tbody>
</table>
Table 2. Mean reaction times and corrected recognition between groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Novel Face (SD)</th>
<th>Familiar Face (SD)</th>
<th>Novel Scene (SD)</th>
<th>Familiar Scene (SD)</th>
<th>d' Corrected Recognition (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High AMBI</td>
<td>1233.69 (151.37)</td>
<td>1178.59 (102.39)</td>
<td>1286.24 (168.55)</td>
<td>1331.76 (149.03)</td>
<td>2.18 (0.60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.74 (0.79)</td>
</tr>
<tr>
<td>Low AMBI</td>
<td>1164.69 (139.42)</td>
<td>1158.80 (137.50)</td>
<td>1183.52 (154.09)</td>
<td>1254.12 (121.09)</td>
<td>2.67 (0.84)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.25 (0.65)</td>
</tr>
</tbody>
</table>
Table 3. Differentially active regions from Whole-brain Group Analysis.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>R/L</th>
<th>Brain region (Brodmann Area)</th>
<th>Max t coordinate (x,y,z)</th>
<th>Volume</th>
<th>Max t value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Familiar Face &gt; Novel Face</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High AMBI</td>
<td>L</td>
<td>Cerebellum IV-IX</td>
<td>-17, -29, -42</td>
<td>13115</td>
<td>7.461</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>Cingulate Cortex (23)</td>
<td>1, -20, 27</td>
<td>2425</td>
<td>6.376</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>Premotor cortex (6)</td>
<td>-12, -11, 67</td>
<td>1624</td>
<td>-7.043</td>
</tr>
<tr>
<td>Low AMBI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Familiar Scene &gt; Novel Scene</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High AMBI</td>
<td>L</td>
<td>Visual cortex prefrontal</td>
<td>0, -85, -15</td>
<td>2417</td>
<td>6.15</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>Dorsolateral prefrontal</td>
<td>-5, 57, 22</td>
<td>2151</td>
<td>-5.366</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>Caudate</td>
<td>-16, 21, 9</td>
<td>1605</td>
<td>-5.477</td>
</tr>
<tr>
<td>Low AMBI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NO CLUSTERS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Samples of face [179] and scene stimuli [180].
Figure 2. Recognition accuracy of stimuli by group. A significant interaction between group and stimulus revealed that those scoring high on AMBI were better at scene recognition and those scoring low on AMBI were better at face recognition.
Figure 3. Brain activity for high AMBI and low AMBI groups. Higher activity to the familiar than novel faces (indicated in orange) was present for the cerebellum from left lobule IV-IX in high AMBI, but not for low AMBI (x= -17, y= -29, z= -42 shown here). Additionally, the dorsolateral prefrontal cortex was more active to novel than familiar scenes (indicated in blue) in high AMBI but no significant difference was found in low AMBI (x= -5, x= 57, x= 22 shown here).
Conclusion

Behavioral inhibition is established as a risk factor for the development of clinical anxiety disorders. However, the underlying neural substrates involved are largely unknown. Research dating back 60 years demonstrates a relationship between eyeblink classical conditioning and anxious traits [189, 190]. These findings have been replicated recently [28, 29], yet the essential circuitry underlying the observed individual differences is still unidentified. The present work sought to first determine the best self-report measure for assessing individual differences in behavioral inhibition, and then apply that measure to examine the cerebellar role in behavioral inhibition. Combined, the results contribute to our understanding of the role of cerebellar functioning in behavioral inhibition.

I. Classical conditioning

Learning approaches to the development of anxiety disorders have been central in empirical perspectives. In 1920, Watson and Rayner demonstrated that specific phobias could be classically conditioned [191]. Since then, research in anxiety disorders has been dominated by learning approaches that blur the line between fear and anxiety [192].

An alternative theoretical perspective combines learning with other risk factors, forming a stress-diathesis approach to the development of anxiety [1]. Classic work assessing eyeblink conditioning in non-clinical populations has been supported by recent follow-up studies [28, 29, 76]. Compared to fear conditioning, eyeblink conditioning is a relatively benign paradigm, combining a neutral tone stimulus with a puff of air to the eye. A demonstration of individual differences in a non-clinical sample is powerful as it suggests enhanced learning reaches beyond threatening stimuli, to possibly all stimuli in the environment. Therefore, it is possible
that the fundamental way an individual processes their environment is different in those vulnerable to, or diagnosed with, clinical anxiety disorders. Given the implications of this suggestion, it is unfortunate that there have been very few studies following up on these findings.

Recent work uses multiple measures of behavioral inhibition and anxiety vulnerability. Some studies report findings in terms of retrospective measures alone [28], some use only adult measures [65, 76], and still others use a combination of measures [29, 37]. A within-subjects comparison of the efficacy of these measures to differentiate acquisition would indicate which one bridges the gap between self-report of behavioral inhibition and learning on a classical conditioning paradigm. To this effect, study 1 was designed to 1) replicate recent research demonstrating facilitated acquisition in behaviorally inhibited participants in a large-scale healthy college-aged sample and 2) directly compare the efficacy of measures of behavioral inhibition and anxiety vulnerability to differentiate learning in eyeblink conditioning. Out of the six self-report measures assessed, results indicated that groups created using the Adult Measure of Behavioural Inhibition and the Trait scale of the STAI demonstrated significant differences in learning. A drawback of these scales is that they do not have any published cutoffs, therefore we chose to use the median split of the sample. This grouping method leaves open the possibility that those scoring around the median could be classified as high or low in different samples. A difficulty arises if these individuals are driving the observed differences. To assess this possibility, we analyzed those who scored extremely high on the AMBI and Trait scales. We found that those who scored in the highest 1/3 still showed significant differences in CR acquisition compared to the rest of the sample. Enhanced CR acquisition in inhibited individuals suggests that they process their environments differently, with greater
sensitivity to the contingencies surrounding events, even those that would be interpreted as relatively neutral.

Study 2 assessed CR acquisition in an adolescent sample by replicating observations in study 1 and then extending them to learning in a long delay conditioning. The comparison of standard delay to a long CS duration permitted the assessment of neural substrates responsible for acquisition. Here, we observed similar learning differences in an adolescent sample as previously observed in healthy adults, with significantly more CRs in standard compared to long delay. Once again, the standard delay learning curve can be separated into two groups with facilitated learning in the inhibited compared to non-inhibited groups. It is clear that those scoring high on AMBI are driving the observed learning curve, with low scorers demonstrating slower acquisition of the CR. There were no group differences in the long delay condition, with both groups showing what appears to be no learning from the first to last block. As longer CS durations are more prone to capture spontaneous or non-specific blinks we included a long-delay unpaired condition to assess non-specific blinks. Comparison of the paired to unpaired conditioning indicates that there is some degree of learning taking place in the long-delay paired condition. However, whatever is learned about the relationship between the tone and airpuff takes place in the first block of trials and is acquired no further. This is supported by a follow up analysis assessing average number of CRs for the paired and unpaired 1000-ms condition, see Figure C.1, and by repeated measures ANOVA revealing a significant interaction of trial x condition, $F(9,630)=2.032$, $p=.034$. 
Together, these eyeblink conditioning studies suggest that AMBI differentiates learning in adolescent and college-age samples. Individual differences in behavioral inhibition are observed in standard delay eyeblink conditioning but not long CS durations, indicating that the cerebellum may be an essential underlying neural substrate in behavioral inhibition.

II. Individual differences of cerebellar functioning

Work examining neural substrates in behavioral inhibition concentrate primarily on structures related to fear conditioning, such as the amygdala [37, 117, 193]. These studies often overlook the cerebellum, which is in a position to modulate processes implicated in behavioral inhibition. Given facilitated learning in inhibited participants in a cerebellar-dependent associative learning task, study 3 was
designed to assess cerebellar connectivity with intrinsic connectivity networks responsible for executive functioning and attention.

Previous research has demonstrated distinct contributions of specific regions of the cerebellum to non-motor networks. However, assessments of individual differences in intrinsic connectivity have not extended beyond clinical applications. Furthermore, prior studies looking at the cerebellum have assessed connectivity using broad approaches such as independent component analysis [51] or large-scale masks [52], which require post-hoc interpretation. The present study was designed to replicate previous demonstrations of a cerebellar role in resting-state functional networks [51,52] and extend this finding to assessment of individual differences in behavioral inhibition. By seeding at the dlPFC, a widely accepted node of the executive network [45], we assessed the presence of correlating intrinsic activity in the cerebellum. In the present study, it was hypothesized that intrinsic activity of the cerebellum would correlate with the executive network, with greater connectivity in the behaviorally inhibited group.

Consistent with our hypotheses, we found that intrinsic activity of the cerebellum lobule VIII correlated with activity of the left and right dlPFC seed regions. Additionally, correlating activity followed known decussation pathways between the cortex and cerebellum, such that activity in the right cerebellum correlated with activity in the left dlPFC seed, and left cerebellum with the right dlPFC seed regions. These findings show specific co-activation of the dlPFC and cerebellum, supporting the growing theory that the cerebellum is involved in non-motor processes. Of critical importance are the results of the between-group analysis. As this is the first study to our knowledge to compare intrinsic connectivity in behavioral inhibition, we expected to see individual differences in cerebellar connectivity based on previous research in
eyeblink classical conditioning [28,29,76]. Additionally, intrinsic activity in the right cerebellum Crus I correlated with intrinsic activity in the right dlPFC seed region. This finding suggests that the Crus I is important in non-motor processes, and specifically may be related to anxiety vulnerability. This evidence is supported by previous studies in resting state which consistently demonstrate Crus I is involved in non-motor processes [51, 52, 122, 125, 194].

Furthermore, activity in the right dACC correlated with the left dlPFC. Although this finding was not necessarily expected, it comes as no surprise given that the dACC is associated with error detection, anticipation, emotional processes and avoidance [149, 157, 158] – all essential components of behavioral inhibition. These findings fit well into current theories of anxiety vulnerability and suggest intriguing implications for the circuitry underlying behavioral inhibition.

So far, we have established a relationship between behavioral inhibition and performance on a cerebellar-dependent associative learning task, demonstrating that those differences extend to executive intrinsic connectivity networks. Combined, this suggests behavioral inhibition is mediated in part by the cerebellum and not the hippocampus. Given previous research on hippocampal differences as a risk factor for PTSD, a follow-up volumetric analysis was conducted on the sample that received neuroimaging [64]. The anatomical weighted T1 scan was segmented and labeled using a procedure that has been shown to be statistically indistinguishable from manual segmentation procedures [153]. Combined volume of the right and left hippocampi was not different between groups, t(24)= -.721, p=.478. A Pearson correlation further demonstrated no relationship between AMBI scores and hippocampal volume, r(24)= .104, p=.613.
Finally, we assessed specific reactivity of the cerebellum to stimulus presentations. Avoidance of social situations is a core feature of behaviorally inhibited temperament [2]. As such, it is possible that those with behavioral inhibition may show differential reactions to novel and familiar faces. Using this rationale, Blackford and colleagues [37] assessed reactivity of individuals with high and low behavioral inhibition to novel and familiar faces. Although they were concentrating on the amygdala as their ROI, they reported an interesting difference in cerebellar activity, such that those with high self-reported scores of behavioral inhibition demonstrated increased reactivity to novel compared to familiar faces in cerebellum lobule VI, a difference not seen in the low scoring group. Although Blackford did not interpret the implications of this finding, we chose to use it as a springboard to design a neuroimaging study specifically looking at cerebellar differences in behavioral inhibition.

At the outset of this study we expected to see increased cerebellar activity in response to novel faces. While our original hypothesis (generated in light of the Blackford findings) was not supported, we feel these results are an important addition to the current knowledge of the cerebellar role in behavioral inhibition. Many procedural and methodological changes were made from Blackford's original study, making direct comparison between the two studies difficult. Unfortunately, there are no other neuroimaging publications to our knowledge documenting the relationship between behavioral inhibition and the cerebellum, so it is presently unclear precisely what role the cerebellum plays in processing novel and familiar stimuli.

We opted to use a cerebellar-centered approach in imaging and to increase the scope of the study by adding behavioral elements and another type of stimuli. As a manipulation check, we assessed behavioral responses and reactivity to faces and
scenes. Overall, responses to faces were faster than scenes, replicating established claims of the speed and accuracy in human face recognition [182]. Furthermore, the FFA demonstrated sensitivity to the faces and the PPA to the scenes, indicating that our procedure and stimuli were appropriate and effective.

Analysis of the behavioral response produced an interesting interaction of AMBI group and stimulus type. Inhibited participants were better at classifying scenes, and worse at classifying faces than non-inhibited. Unfortunately, this study was not designed to assess strategies in stimulus processing so it is difficult to tease out what factors may be influencing this difference. It is possible that biased processing observed in inhibited individuals affects recognition memory. Although the face stimuli selected are considered to be neutral expression, inhibited individuals may interpret them as negative or threatening [63, 183-187]. Future research using methods such as eye tracking while participants process faces and other stimuli may shed light to different underlying processing strategies in anxiety vulnerability.

Although we expected inhibited individuals to demonstrate increased reactivity to novel faces, we observed increased activity to familiar faces. Cerebellar lobules VI-IX were more active to familiar faces than novel faces. This area overlaps with that reported by Blackford [37], yet we are showing the opposite effect. Given cerebro-cerebellar connectivity it is possible that the cerebellum is not reacting to the novel or familiarity of the stimuli, but to violation of expectation. The cerebellum could be updating input to the cortex about the environment by modulating the signal carried by reciprocal pathways to the cortex. Thus, cerebellar activity may be related to the participant’s expectations. A possible explanation lies in individual differences in timing, which is essential in both motor learning as well as higher cognitive
functions such as stimulus processing, expectations, and attention. Using repetitive transcranial magnetic stimulation to create a virtual “lesion” of selected brain regions, researchers demonstrate that interference of either the cerebellum or dIPFC affect performance on a duration judgment task [195]. Furthermore, researchers have found that the cerebellum is active when expectations are violated, especially temporal expectations [196]. In our study, we used a rapid event-related design that maximizes attention and novelty, as participants do not know when to expect the next stimulus. This paradigm may affect participant’s timing expectations, which may increase expectancy and preparedness in behavioral inhibition. Combined, the manipulation of timing and expectation may alter cerebellar functioning, and affect modulation of the signal from the prefrontal cortex. Rather than only in times of uncertainty, this cascade of events may take place frequently in behaviorally inhibited individuals who are more reactive to the stimuli in their environments [12,13,63]. In this case, increased reactivity to familiar faces may be the result of cerebellar modulation of prefrontal signals, updating higher cognitive regions that the stimulus is familiar, non-threatening, and not worthy of additional action or attention.

Cerebellar contributions to cognitive processes is a complicated interplay of reciprocal loops, inhibitory pathways, and feedback clearly demanding more research into the various situations the cerebellum is active in cognitive functions.

III. Summary

The theory that the cerebellum is involved in higher cognitive processes and emotion is no new concept. Nearly thirty years ago Leiner and colleagues gathered and summarized extensive evidence, proposing a cerebellar role in non-motor processes to little reception [197-199]. Despite mounting evidence, researchers continue to overlook the cerebellum as a factor in cognitive processes. Only recently
has appreciation for the cognitive cerebellum gained ground. For example, the Habas's paper [51] that demonstrated distinct contributions of the cerebellum to the major intrinsic connectivity networks, has been cited nearly as many times in the past year as it had been in the first four years following its publication. Clearly, scientists are beginning to recognize the non-motor aspects of cerebellar functioning.

However, while general acceptance for cognitive functions of the cerebellum is increasing, its role specifically in anxiety disorders has yet to be explored. The current results suggest that the cerebellum is an essential neural substrate in anxiety vulnerability. This is in agreement with previous research in eyeblink conditioning and neuroimaging [28, 29, 37]. The studies presented were designed to specifically assess the cerebellar role in behavioral inhibition, demonstrating that it is the cerebellum, and not the hippocampus, that is mediating individual differences in classical eyeblink conditioning. This behavioral observation was further supported by direct assessment of cerebellar connectivity and reactivity.

As techniques and methods improve, it is clear that no single factor mediates risk for anxiety. Therefore, an understanding of the factors promoting the transition from healthy to clinical anxiety is essential. Behavioral inhibition is a risk factor that is expressed by approximately 15% of the population [11-13]. Combined with statistics of anxiety in the population reaching 25% [67, 68], it is clear that it is necessary to understand the factors contributing to the development of anxiety. Research in clinical populations of anxiety has provided valuable knowledge about the behavioral, psychological, and physiological profiles of anxious individuals. However, this approach comes inevitably after the fact, possibly missing the critical factors in the development of anxiety. Assessment of pre-clinical anxiety permits the exploration of the biological and psychological factors contributing to vulnerability to
anxiety disorders. Here, we demonstrate that the cerebellum may be an essential component in the neural circuitry underlying anxiety vulnerability. Continued research in this area would provide a more complete understanding of the cerebro-cerebellar pathways in humans and may be useful to inform treatment approaches and shed light on the presently unknown neural processes underlying anxiety.
References


