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

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HIGHLIGHTS
- Inhibited individuals demonstrate facilitated learning at standard delay (500-ms) eyeblink conditioning.
- There are no differences in learning between inhibited and non-inhibited individuals at long delay (1000-ms) conditioning.
- Facilitated learning of standard delay eyeblink conditioning supports a cerebellar role in anxiety vulnerability.
- Adolescents demonstrate similar learning in delay and long delay eyeblink conditioning to young adults.

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 50-ms airpuff US. A third group received unpaired training of 1000-ms CS and 50-ms airpuff US. Inhibited individuals showed greater 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 [1,2]. 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 [3]. Recent research suggests that individual differences in learning may also be an important risk factor for anxiety vulnerability [4–6].

Behavioral inhibition (BI) is a personality factor linked to the development of anxiety disorders [7–9]. BI is observable early in life and persists through the lifespan [10]. Individuals with BI demonstrate similar physiological and behavioral profiles as those with clinical anxiety including altered heart rate reactivity [11,12], adrenocortical activity [13], apprehension, withdrawal and avoidance [14,15].

Avoidance is a key symptom of both behavioral inhibition and clinical anxiety [12,16,17], 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. This theory is supported by the observation of individual differences in learning in both operant and classical conditioning avoidance paradigms [4–6, 8, 18, 19]. Although operant paradigms may seem more suited to the high cognitive processes typically associated with avoidance, eyelink classical conditioning is an established and reliable model for understanding human learning. In classical eyelink conditioning, a conditioned stimulus (CS) and unconditional stimulus (US) are repeatedly paired, resulting in the acquisition of a conditioned response (CR), the measure of learning. Eyelink classical conditioning is also one of the few preparations that has an advanced understanding of the neural substrates underlying acquisition with general consensus that the cerebellum is both necessary and sufficient to acquire standard delay eyelink conditioning [20–23]. 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; [4]), by altering the reinforcement schedule [6], or by adjusting the contingencies between the CS and US such as in long delay and trace paradigms [24].

The effects of development on the acquisition of the conditioned eyelink response have been assessed at length in infants and adults, largely overlooking the period of adolescence. Eyelink conditioning has been used to demarcate the development of key underlying neural substrates in infants and young children [25–28]. Research in adults concentrates on aging to understand the neurobiology underlying age-related memory disorders [29–32]. Considering that adolescence is a critical period in refining cortical connections as well as for the development of psychopathologies such as anxiety and schizophrenia [33–35] understanding how eyelink conditioning is affected may shed important light on underlying neural networks.

Using eyelink 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, demonstrated significantly faster learning in a standard delay (500-ms) conditioning paradigm [4]. 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 the present study was twofold: First, we utilized a basic science approach to assess acquisition of standard delay eyelink 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 long delay (1000-ms) CS durations. Longer CS durations have slower ontogenetic development [27] and can be more difficult to acquire than standard delay durations [24, 27, 36]. 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 [24, 36].

Although delay conditioning is typically considered as cerebellar and trace conditioning as hippocampal, the dichotomy between the two paradigms is not so clear-cut. Evidence suggests the hippocampus is involved during delay eyelink conditioning. Pyramidal neurons in the hippocampus show increased responding during the CS period in conjunction with development of the behavioral CR, declining with continued training [37]. Neuroimaging studies reflect a similar pattern of activity in the hippocampus during delay eyelink acquisition [38, 39], suggesting that although the hippocampus is not essential in standard delay eyelink acquisition it still plays a role under normal learning circumstances.

Although the hippocampus is involved during normal learning, hippocampal lesion typically does not affect delay eyelink conditioning and can actually enhance learning, suggesting the hippocampus may interfere with cerebellar functioning [40]. This is supported by studies assessing acquisition of eyelink conditioning following hippocampal lesion in delay, long delay, and trace paradigms. Breylin et al. [24] found that unlesioned rats took longer to acquire long delay eyelink conditioning than standard delay, with acquisition rates similar to that observed in trace conditioning. Additionally, acquisition of long delay eyelink conditioning was significantly slower in the hippocampal lesioned rats, suggesting that the hippocampus plays a role in its acquisition. Developmental work supports this finding, demonstrating that infant rats can acquire short-delay conditioning, but are impaired at acquiring both long-delay and trace conditioning, which emerges in parallel later in development [41, 42]. Therefore, long CS durations provide a useful paradigm to explore the influence of the hippocampus on acquisition without altering the conditioning parameters as drastically as trace conditioning would.

Comparing acquisition of eyelink conditioning in long and short delays provides a means for understanding whether the neural basis for enhanced acquisition in BI is primarily through reduced hippocampal involvement. Reduced hippocampal involvement in BI would be evident as faster short delay (as previously observed; [5]), concomitant with impaired long delay. If BI is associated with faster acquisition at both short and long delay, this finding suggests that either the facilitation is primarily mediated through the essential cerebellum brainstem/cerebellar circuitry or through those modulatory sites whose influence are in the same direction (accentuation of excitatory influence or diminution of inhibitory influence).

2. Materials and methods

2.1. Participants

168 participants were recruited from a local public high school in New Jersey. Participant’s ages ranged from 13 to 19 (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; [43]), and the State/Trait Anxiety Inventory (STAI; [44]).

The Adult and Retrospective Measure of Behavioural Inhibition (AMBI/RMBI; [43]) 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 [43]. Scores on the 16-item AMBI range from 0 to 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 to 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 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” [44].

2.3. Eyeblink conditioning

Eyeblink conditioning apparatus and procedures was the same as previously described [45]. 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 82 dB 1200 Hz 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 1 Hz and 30 Hz, 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 min, during which participants watched a silent movie (Planet Earth) to reduce boredom and maintain a forward-facing gaze.

2.4. Procedure

Following consent, individuals were randomly 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 120 explicitly unpaired CS and US trials 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 to 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 occurring immediately before the onset of the CS [45]. To maintain continuity between conditions, responses were only counted as a CR if it occurred within the 500-ms prior to the onset of the US. 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 [4–6,18].

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, X2(1, N = 123) = .659, p = .417. Given previous research with these measures in eyeblink conditioning [5], 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 on 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 (Block) mixed measures ANOVA. A Group x Block interaction, F(5,480) = 5.039, p < .01, indicates differences in overall learning between groups. Post-hoc repeated measures ANOVA’s revealed that significant learning occurred in the 500-ms condition, F(5,240) = 13.424, p < .01, but not in the 1000-ms condition, F(5,240) = 0.671, p = .646, see Fig. 1. There was also a significant

<table>
<thead>
<tr>
<th>Condition</th>
<th>Inhibited High AMBI (&gt;15.5)</th>
<th>Non-inhibited Low AMBI (&lt;15.5)</th>
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<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>12</td>
<td>19.91 (2.80)</td>
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AMBI = Adult Measure of Behavioural Inhibition. SD = standard deviation.

Fig. 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 was present in the 500-ms CS duration, but not the 1000-ms CS duration, as evidenced by a Group x Block interaction. Follow-up post-hoc tests revealed that significant learning over the acquisition period occurred in the 500-ms condition, but not the 1000-ms condition. Error bars represent the standard error of the mean.
between-subjects Group x Condition interaction, F(1.96) = 4.788, p = .031. Follow-up independent samples t-tests indicated that high AMBI had more overall CR responses than low AMBI. Error bars represent standard error of the mean.

There was no difference of initial UR amplitudes between the conditions, F(1.99) = 1.388, p = .242, or between the AMBI groups, F(1.99) = 636, p = .427. Analysis of the other self-report measures used (RMBI, State, and Trait) revealed no significant learning differences, all p’s > .068. Finally, there were no significant sex differences in acquisition, all p’s > .0328.

Longer CS durations are prone to capture a greater number of non-specific blinks. To assess the prevalence of non-specific blinks at 1000-ms we compared the CS-US paired condition to the explicitly unpaired condition that presented the CS and US stimuli separately. Using the CS alone trials from the 1000-ms unpaired condition, A 2 (Group: High/Low) x 2 (Condition: Paired/Unpaired) x 6 (Block) mixed measures ANOVA revealed a between-subjects effect of condition, F(1.69) = 17.392, p < .01, indicating a greater overall number of CRs in the 1000-ms paired condition, yet no learning over the acquisition blocks.

A follow-up analysis of the first 10 trials for each condition using a 3 (Condition 500-ms paired/1000-ms Paired/1000-ms Unpaired) x 10 (Trial) mixed measures ANOVA revealed a significant main effect of trial, F(9,1080) = 2.782, p = .003 and a significant main effect of condition, F(1,120) = 8.431, p < .001 (Fig. 3). Comparison of mean responses over the first block revealed that the 500-ms and 1000-ms paired learning groups produced more CRs (40.8% and 32.6%, respectively) than the unpaired group (17.4%). However, only the 500-ms paired group demonstrated significant acquisition, F(9,441) = 2.909, p = .002, all other p’s > .091. Finally, assessment of the US alone trials in the unpaired condition demonstrated no differences between responses made to the CS alone to those made prior to the onset of the US, F(5,220) = .613, p = .690, suggesting that the CR rate for the unpaired condition is simply a measure of spontaneous blinking during eyeblink classical conditioning.

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 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, learning was 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 [4–6]. Learning was significantly reduced in the 1000-ms paired CS condition, similar to what is observed in Herbert et al. [27] in adult long delay with a CS duration of 1250-ms. These findings are consistent with previous research demonstrating acquisition under long delay is more
similar to trace than standard delay procedures in infant rats [42] and those with hippocampal lesions [24]. Although it should be noted that there have been some reports of the opposite effect, suggesting similarities between long-delay and standard delay conditioning using young adult rabbits [46,47]. It is clear that continued research using long-delay methods is necessary to fully understand the essential underlying neural substrates and behavioral outcomes specifically in humans.

Previously, we demonstrated that those who score high on AMBI acquire standard delay eyelink conditioning to a greater extent [5]. 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 compared acquisition of standard delay to a longer CS duration. Differences in learning at these two CS durations shed light on underlying neural substrates influencing acquisition.

Hippocampal dysfunction in inhibited individuals is one viable explanation for previously observed learning differences in standard eyelink conditioning. Previous research indicates the hippocampus may be involved in risk for anxiety in terms of structure [48], function [49], and behavior [19]. Reduced hippocampal activity in the high AMBI group could enhance learning in standard delay (500-ms) conditioning. Alternatively, cerebellar activity could be altered. Recent neuroimaging studies indicate increased cerebellar activity in Lobule VI of inhibited individuals to novel faces [50], as well as during eyelink conditioning [38]. It is possible that individual differences in activity in the Lobule VI region are contributing to facilitated eyelink conditioning in high AMBI. Here, we found that those with high AMBI scores had more CRs 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, suggesting hippocampal dysfunction is not mediating observed individual differences.

The Adult Measure of Behavioural Inhibition [43] is a useful tool in differentiating classical eyelink conditioning. Here, we demonstrate that it is sensitive to associative learning differences in an adolescent sample, similar to previous observations in college-age students. The AMBI has not been used previously with an adolescent sample, therefore it is unclear precisely how valid the scores are. Comparison to unpublished data from the same population indicates that scores in this study were similar to those previously observed, t(461) = −2.887, p = .012. However, continued research assessing the validity of the AMBI measure in general, and specifically in adolescent populations is warranted.

Future research in this area will contribute to understanding the role of the cerebellum in anxiety vulnerability. In recent years interest has increased in understanding the higher cognitive capacities of the cerebellum (for a review see [51]). 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 [52,53]. 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 [54–56]. 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 [57–59]. Finally, task-based neuroimaging studies have reported increased cerebellar activity in emotional, language, memory and executive tasks [59].

4.1. Conclusions

Together, these studies make a clear case that the cerebellum is involved in higher cognitive functions, including those related to emotion and executive functioning. Cerebellar dysfunction might 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.

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