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Delay Eyeblink Classical Conditioning is Impaired in Fragile X Syndrome

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Abstract

We examined 400 ms delay eyeblink classical conditioning in 20 participants with Fragile X syndrome ages 17–77 years, and 20 age-matched, healthy control participants. The Fragile X group demonstrated impaired learning and abnormal conditioned response timing. Adults with Fragile X (n=16) were also tested at two successive 12-month follow-up sessions to examine reacquisition and long-term retention. Participants in groups older and younger than 45 years demonstrated significant learning during each reacquisition session. Younger participants demonstrated greater retention of the CS/US association at each follow-up session than older participants. Fragile X impairs the acquisition and timing of conditioned eyeblink responses, but with repeated training adults with Fragile X syndrome show significant plasticity.

Keywords

cerebellum; mental retardation; aging; longitudinal; FMRP

Delay eyeblink classical conditioning is a cerebellum-dependent form of simple associative learning (Christian & Thompson, 2003). This form of associative learning has utility in basic neuroscience research concerned with brain mechanisms of learning and memory (Woodruff-Pak & Steinmetz, 2000a, 2000b), and is impacted by processes of normal aging (e.g., Woodruff-Pak & Thompson, 1988) and psychopathology (Brown, Kieffaber, Carroll, Vohs, Tracy, Shekhar et al., 2005; Greer, Trivedi & Thompson, 2005; Steinmetz, Tracy & Green, 2001). Fragile X syndrome (FX) is a common heritable form of mental retardation characterized by cognitive and behavioral impairments that may be partly attributable to cerebellar dysfunction. The purpose of this investigation is to assess cerebellar functioning with delay eyeblink conditioning in a large sample of human participants with FX, and to determine whether or not participants with FX are susceptible to the effects of normal aging on the acquisition of conditioned eyeblink responding.

Delay eyeblink conditioning is an associative learning task in which the stimuli that are associated are temporally contiguous. A neutral conditioned stimulus (CS) is associated with a reflex-eliciting unconditioned stimulus (US) via paired temporally contiguous presentation and simultaneous cotermination of the stimuli. Repeated exposure to CS/US pairings stimulates cerebellar plasticity (Kleim, Freeman, Bruneau, Nolan, Cooper, Zook & Walters, 2002) that

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ultimately controls the emergence of a conditioned response (CR) that is elicited by the CS in the absence of the US. The frequency (i.e., percentage of trials that produce a CR) with which the CS elicits a CR is one of the primary dependent variables used to assess learning. Topographical analyses such as examination of the CR waveform (i.e., amplitude) and timing characteristics (i.e., response onset and/or latency to peak amplitude) are also used to assess additional cognitive processes transpiring during delay eyeblink conditioning.

The cerebellar cortex and deep nuclei are involved in numerous aspects of delay eyeblink conditioning. Human patients with cerebellar lesions located ipsilateral to the conditioned eye are significantly impaired at acquiring delay eyeblink conditioning (Woodruff-Pak, Papka & Ivry, 1996). Patients with selective lesions of the cerebellar cortex are also impaired at acquiring the task and demonstrate CRs with significantly premature peak latency (Gerwig, Hajjar, Dimitrova, Maschke, Kolb, Frings et al., 2005). Volume of the cerebellar cortex is significantly positively correlated with acquisition of delay eyeblink conditioning in healthy adults (Woodruff-Pak, Vogel, Ewers, Coffey, Boyko & Lemieux, 2000), and functional neuroimaging has revealed cerebellar activation in humans (Blaxton, Zeffiro, Gabrieli, Bookheimer, Carillo, Theodore & Disterhoft, 1996; Logan & Grafton, 1995) and rabbits (Miller, Chen, Tom, Weiss, Disterhoft & Wyrwicz, 2003) during delay eyeblink conditioning. In addition, the cerebellar interpositus (i.e., globose in humans) nucleus is essential for acquisition, expression and retention of conditioned eyeblink responding (Krupa & Thompson, 1997), and may be the locus of plasticity for permanent storage of the conditioned association (Kleim et al., 2002).

FX is a genetic disorder that affects approximately 1 in 4000 males and 1 in 8000 females (Turner, Webb, Wake & Robinson, 1996). Symptoms include mild to severe mental retardation manifest as various cognitive and behavioral deficits, poor motor coordination, and abnormal physical characteristics. The disorder is caused by mutation of the FMR1 gene on the X chromosome, which codes for expression of the Fragile X mental retardation protein (FMRP) throughout the brain. Expression of FMRP is especially prevalent in the hippocampus and cerebellum (Hinds, Ashley, Sutcliffe, Nelson, Warren, Housman & Schalling, 1993). The mutation itself is defined as excessive repeats (i.e., greater than 200) of the CGG trinucleotide (Verkerk et al., 1991). Premutation carriers having more than 50 and less than 200 repeats (less than 50 repeats is normally found in healthy individuals) are at risk for developing some of the symptoms of the full blown disorder, especially tremor ataxia (Hagerman & Hagerman, 2004), suggesting that FMRP is especially important in the control of motoric responding. Mutation of the FMR1 gene and the subsequently reduced expression of FMRP is associated with numerous morphological and functional abnormalities of the cerebellum that are related to FX (Huber, 2006). Clinical research reports evidence of significantly abnormal cerebellar morphology (i.e., reduced cerebellar volume) that is correlated with cognitive assessments of executive functioning (i.e., Wisconsin Card Sorting Task) and full-scale IQ in individuals with FX (Mosotofsky, Mazzocco, Aakalu, Warsofsky, Denckla & Reiss, 1998).

The known genetic etiology of the disorder provides an equitable basis for the development of a gene knockout animal model in which all aspects of the underlying neurological impairment may be studied. Hanson & Madison (2007) found that FMR1 influences the degree of synaptic connectivity in vitro brain slices from FMR1 knockout mice. Irwin, Galvez & Greenough (2000) reported dendritic spine structural anomalies in FMR1 knockout mice. Huber, Gallagher, Warren and Bear (2002) reported enhanced long-term depression (LTD), a form of synaptic plasticity mediating learning and memory, in hippocampal slices taken from FMR1 knockout mice.

Koekkoek and colleagues (2005) reported an extensive study of the FMR1 knockout mouse model of FX and found several significant morphological, functional, and behavioral symptoms of the genetic mutation that likely account for some of the cognitive and behavioral

dysfunction observed in relation to FX in humans. Specifically, findings in the data gathered from FMR1 knockout mice include elongated dendritic spines, enhanced long-term depression at parallel fiber-to-Purkinje neuron synapses, and impaired acquisition of delay eyeblink conditioning. Moreover, Koekkoek and colleagues reported that humans diagnosed with FX also demonstrated impaired delay eyeblink conditioning that resembled the impairment observed in the knockout mice, although both the sample of behavior and population sample were narrowly limited. Nonetheless, such findings provide evidence of cerebellar dysfunction in individuals with FX that validate the animal model currently under investigation. However, the findings from any genetic animal models of psychopathology are void if the behavioral deficits inadequately replicate the human condition. As such, the focus of this paper is the eyeblink conditioning performance of human subjects with FX across the adult lifespan from age 17 to 77 years.

A related line of clinical research includes several reports that autism, a developmental disorder with behavioral and neurobiological parallels to FX (Hagerman, 2006), is associated with abnormal delay eyeblink classical conditioning in humans (Sears, Finn & Steinmetz, 1994) as well as teratogenic animal models (Stanton, Peloso, Brown & Rodier, 2006). More specifically, individuals with autism demonstrated enhanced (i.e., rapid acquisition) delay eyeblink conditioning, as well as disorganized timing manifest as significantly early response onset and premature peak latency (Sears et al., 1994). These findings of enhanced acquisition and disorganized timing have been replicated in a rodent model of autism resulting from prenatal valproic acid exposure (Arndt, Chadman, Watson, Tsang, Peloso, Rodier & Stanton, 2008; Murawski, Brown & Stanton, 2008; Rodier, Ingram, Tisdale, Nelson & Romano, 1996). While the autism diagnosis and FMRP are not directly linked, autism and FX are linked via common symptoms, high rates of comorbidity, and the implication of cerebellar dysfunction (Hagerman, 2006). Thus, assessment of eyeblink conditioning in a sample of 20 adults with FX also has potential to identify neurobiological commonalities between autism and FX.

Study 1

Method

Participants—Forty participants ranging in age from 17-77 years comprised of individuals diagnosed with FX ($n = 20$; mean age = 45.92, $SD = 21.3$) and healthy age-matched control participants ($n = 20$; mean age = 45.79, $SD = 21.74$) participated in the study. All 20 adults with FX (17 male; 3 female) were confirmed to have that disorder by cytogenetic analysis with percent fragility ranging from 5% to 45% with a mean of 18%. FX participants were clients of Elwyn, a regional service provider of institutionalized care for individuals with mental disabilities, and medications varied across participants precluding analyses of pharmacological effects. Medications included phenytoin, L-thyroxine, hydrochlorthiazide, nifedipine, and insulin. All participants were tested with the same apparatus. Normal participants ($n=20$; 15 male; 5 female) were recruited by letters and posted signs to students and staff at Temple University and a health maintenance organization affiliated with the Philadelphia Geriatric Center. All participants were assessed for eye size and baseline blink rate. These data, along with other participant characteristics are presented in Table 1.

Apparatus—The eyeblink conditioning apparatus is commercially available from San Diego Instruments, and consisted of a portable, automated system with an infrared eyeblink detector and airpuff jet attached to headgear and input to an interface box containing a microprocessor and a miniature air compressor. The interface box was interactive with a computer. The headgear had an adjustable headband holding the airpuff jet and infrared eyeblink monitor. Filtered air was compressed in the interface box and delivered through a jet that was set approximately 2 cm from the cornea.

The voltage from the infrared device was amplified and differentiated; eyelid data were transferred to the computer for storage and analysis. From the speaker of the interface box, a 1000Hz, 80 dB SPL tone CS was delivered through headphones. Air for the corneal airpuff US was compressed in the interface box, filtered, and delivered at a pressure of 5-10 psi. The timing of stimulus presentations was controlled by the microprocessor in the interface box. Figure 1 depicts the sequence of events during a 400 ms delay eyeblink conditioning trial, and includes a sample waveform with the data points used in analysis marked to clarify the methods employed in this study.

Procedure—All participants were exposed to at least 72 trials of 400 ms delay eyeblink conditioning. Some FX participants were exposed to as many as 90 trials so that 72 analyzable trials could be obtained (un-analyzable trials included participants rubbing their eye, shaking their head, holding eyes closed, etc). The conditioning paradigm was administered in 8 blocks of 9 trials per block for all participants, with additional trials for the FX group (10 blocks of 9 trials). The first trial of each block was a CS-alone trial and the remaining trials were paired CS-US trials. The US was a corneal airpuff (5-10 psi, 100 ms duration) and the CS was a tone (80 dB, 1kHz, 500 ms duration). On paired trials CS onset preceded the US by 400 ms and the stimuli co-terminated. Participants watched a silent movie (Gold Rush or The Three Stooges) throughout the session to alleviate ennui. Participants were asked to remain alert, attend to the tones, and instructed as follows:

Please make yourself comfortable and relax. From time to time you will hear some tones and feel some mild puffs of air in your eye. If you feel like blinking, please do so. Just let your natural reactions take over.

At that point, the experimenter answered any questions the participant had about the procedure and then initiated the conditioning trials.

Analysis—All analyses were conducted using SPSS 14.0. Data from paired trials only were analyzed because CS-alone trials ($n=8$) represent a limited sample from which to draw meaningful conclusions. While the CR topography is not contaminated by the UR on CS-alone test trials, the focus of this paper is the differential acquisition of a conditioned response and subsequent modulation of its temporal characteristics in participants with FX, rather than memory testing for acquired CRs. As such, the test trial data have been excluded from analyses thereby precluding discussion of the theoretical importance of so few observations of behavior, as well as the theoretical significance of long latency CRs occurring during the UR period on trials without a US.

Bad trials (spike in amplitude prior to CS presentation) and alpha responses occurring 75 ms or less after CS presentation (Gormezano, 1966) were excluded from analyses. Missing onset, CR peak latency, and CR peak amplitude data values for FX participants (less than 15% across all FX participants) were replaced with the session mean for that individual subject to preserve power in our statistical analyses. No data were missing for the analysis of CR percentage, and no data were missing from the control participants for any dependent measure. Data from one FX participant could only be analyzed for percentage CRs; the matched control participant was also excluded from analyses other than percentage CRs. All significance tests are two-tailed with alpha set at .05 unless otherwise noted. Violations of the sphericity assumption for repeated measures ANOVA required interpretation of adjusted p -values. For each adjustment the true value of ϵ is reported.

Results

The correlation ($r = .327$) between percent fragility from the diagnostic cytogenetic analysis of participants in the FX group and percentage of CRs for the FX group was non-significant (p

> .05, $n = 11$). However, the FX group showed impaired acquisition and abnormal timing characteristics compared to the age-matched healthy control group. Analyses of each dependent measure collected during eyeblink conditioning is reported below in a 2 (Group: FX vs. Control) $\times$ 8 (Training Block) repeated measures ANOVA. Group is the between subjects variable, and Training Block is the within subject variable. The statistical analyses reported below reveal several main effects for Training Block showing that participants from either group were able to acquire the conditioned association, while acquisition of such associative responding was impaired in the FX group. In addition, results of the statistical analyses revealed only minor, non-significant group differences in UR characteristics, including amplitude and peak latency. Such findings rule out potential confounds stemming from the (dys)functionality of the motor pathway, and emphasizes the existence of an associative learning deficit in the FX group.

Percentage CRs—The ANOVA with percentage of paired CRs as the dependent variable revealed significant main effects of Group $F(1,38) = 4.994$, $p = .031$, and Training Block $F(1,6.004) = 5.44$, $p < .001$ ($epsilon = .858$). The average CR percentage was significantly reduced in the FX group (37.8%) compared to the control group (54.9%). Over training sessions CR percentage increased for both groups indicating that both FX and control participants acquired the CS/US association, and that the strength of association increased with repeated exposure (Figure 2). The Group $\times$ Training Block interaction was non-significant.

Response Onset—Response onset is the timing of the initial increase in amplitude that defines a motor response regardless of its classification as UR or CR. CRs and URs are not separated in this analysis in order to demonstrate the modulation of response timing as a result of experience with the contingency that corresponds to delay eyeblink classical conditioning. It is not a measure of CR onset, but rather a measure of how the motor response shifts from a reflexive response occurring after presentation of the US to a learned response that occurs prior to presentation of the US. If the motor response does not onset prior to presentation of the US then it demonstrates a deficit in the ability to produce a functionally adaptive motor response that is controlled by the environmental contingencies corresponding to eyeblink conditioning. The ANOVA with Response Onset as the dependent variable revealed a significant main effect of Group $F(1,36) = 8.454$, $p = .006$ and a significant main effect of Training Block $F(1,7) = 4.392$, $p < .001$. The main effect of Training Block is depicted in the change in motor onset timing from Block 1 through Block 8 and shows that both groups modulated Response Onset over training blocks (Figure 3). FX participants demonstrated significantly delayed Response Onset (446 ms) compared to the control group (372 ms). This may reflect a deficit in acquisition consistent with the impaired CR percentage noted above. The interaction was non-significant.

CR peak latency—In contrast to Response Onset, only trials that produced a CR were included in the analysis of CR peak latency. CR peak latency indicates the acquisition of temporal information that optimizes the adaptive function of eyeblink conditioning. CRs that peak closer in time to onset of the US are well-timed and considered more adaptive. For the analysis reported below, CR peak latency refers to the peak of the waveform representing the CR during the interval between presentation of the CS and subsequent presentation of the US. As such, the data does not represent the timing of the peak of the CR waveform per se, but represents the timing of the peak of the CR waveform that was observable prior to presentation of the US. The ANOVA with CR peak latency as the dependent variable revealed a significant Group $\times$ Training Block interaction, $F(1,5.332) = 2.253$, $p = .047$ ($epsilon = .762$). The interaction indicates a different effect of Group on CR peak latency dependent on Training Block. FX and control participants shifted the timing of their responses in opposite directions throughout the procedure, with FX participants shifting the timing of the peak amplitude of

their CRs in a maladaptive direction (Figure 4). The main effects of block and group were non-significant.

CR amplitude—CR amplitude is measure as the peak height of the waveform for responses designated as a CR based on the timing of its onset. Only trials that produced a CR were included in the analysis of CR amplitude. The measurements for amplitude used in the analysis below are not measures of the peak amplitude of the CR per se, but are measures of the peak amplitude of the CR during the interval between CS presentation and US presentation. The ANOVA with CR amplitude as the dependent variable revealed a significant main effect of Training Block $F(1,5.041) = 5.681, p < .001$ ($\epsilonpsilon = .720$). The main effect of Training Block indicates both groups increased CR amplitude throughout the procedure. The main effect of Group, and the Group x Training Block interaction were non-significant, indicating similar response amplitude across participants over each Training Block.

UR characteristics—Analysis of the UR characteristics revealed a non-significant main effect for Group for each dependent measure, and non-significant interactions. The non-significant group differences for UR characteristics emphasize group differences in learning and adaptive functioning associated with the CR, and suggests that such effects are not attributable to underlying motor impairment or dysfunction of the UR pathway. The ANOVA with UR peak latency (i.e., the peak of the waveform during UR period associated with a response onset that is unique from that of the CR) as the dependent variable revealed a significant main effect of Training Block $F(1,3.913) = 2.562, p = .042$ ($\epsilonpsilon = .559$). This finding indicates that the latency to the peak of the UR waveform decreased with repeated exposure to the procedure. The between subjects main effect of Group and the interaction were non-significant suggesting that this modulation of reflex timing occurred similarly for participants in both groups across training blocks. Additional analyses of UR characteristics yielded statistically non-significant interaction and main effects.

Discussion

Fragile X syndrome is associated with impaired delay eyeblink classical conditioning. The FX group demonstrated significantly impaired acquisition compared to the control group, and response timing characteristics for both response onset and CR peak latency that significantly deviated from the timing characteristics demonstrated by the normal control participants. The effect of Group on CR-related performance variables is neither attributable to nor confounded by the presence of a deficient motor pathway in the FX group. These conclusions are bolstered by the additional findings that the UR characteristics did not differ significantly between the FX and control groups. Whereas UR peak latency differed as a function of Training Block, it failed to differentiate the groups and so cannot account for group differences in timing characteristics associated with the CR.

The results of Study 1 show that simple associative learning processes controlled by the cerebellum are impaired in individuals with FX compared to healthy, age-matched control participants. The cerebellar dysfunction in FX extends to adaptive behavioral processes as indicated by the significantly premature peak latency of conditioned eyeblink responses in the FX group. Analysis of the UR characteristics indicates that the motor pathway is not impaired, strengthening the conclusion that associative learning processes are disrupted in FX. Our findings are in partial agreement with the currently available literature in that neural processes involved in acquisition of the CS/US association are impaired in FX, but we are the first report of impaired CR timing in FX.

The source of impairment is likely attributable to dysfunction in the cerebellar cortex rather than the deep nuclei. The deep nuclei, especially the globose (analogue of the rabbit and rodent

interpositus) nucleus, are essential for acquisition, expression and retention of conditioned eyeblink responding (Krupa & Thompson, 1997). Subjects would be unable to acquire any conditioned responses if the deep nuclei were severely damaged or if the neurobiological mechanisms of plasticity were altered. The cerebellar cortex, in contrast, is not an essential component of the eyeblink conditioning circuit (Lavond & Steinmetz, 1989), but rather is involved in normal acquisition of associative conditioning when the CS and US are temporally contiguous (Kishimoto, Fujimichi, Araishi, Kawahara, Kano, Aiba & Kirino, 2002). Also, Gerwig et al. (2005) found that human subjects with selective lesions of the cerebellar cortex that spared the deep nuclei demonstrated premature CR peak latency, in addition to impaired acquisition. The subjects with FX in our study demonstrated significant albeit impaired acquisition in conjunction with premature peak latency. Our findings suggest that FX involves cerebellar cortical dysfunction that is similar to the dysfunction observed in patients with cerebellar cortical lesions.

Purkinje neurons are the sole output of the cerebellar cortex, although they are not the only neurons that comprise the cerebellar cortex. Koekkoek and colleagues examined the role of Purkinje neurons in delay eyeblink conditioning in the knockout mouse model of FX. They created a strain of mice in which FMR1 was silenced only in Purkinje neurons, allowing for observations of differential effects of selective versus global deficiencies in FMRP expression. The results of their experiments indirectly implicate Purkinje neurons in the cerebellar dysfunction that characterizes FX in that the neurons lacking FMRP demonstrated normal firing patterns but abnormal plasticity. As such, the Purkinje neurons can effectively stimulate the deep nuclei, but cannot adapt to effectively encode the temporal relationship between incoming CS and US information. The result is impaired acquisition and disrupted timing. However, Koekkoek et al. did not find significantly premature CR peak latency as we reported above. This discrepancy may be due to the small sample size or limited set of behavioral observations employed in their study.

There are numerous types of acquired CRs that are each defined by their temporal and topographical characteristics. While each is a legitimate acquired response, not all types of CRs are considered adaptive. An adaptive response is characterized by late onset and a subsequently rapid peak near presentation of US. Closing the targeted eye early (early onset) and sustaining eyelid extension is not an adaptive response, although it is learned and is probably voluntary, because it leaves the organism vulnerable to additional impending aversive stimulation signaled through the visual modality. Moreover, early onset CRs preclude modulation of response frequency and timing that are dependent on conditional or contextual cues, regardless of their modality of presentation. CRs with a late onset are ineffective in protecting the eye from the US unless the response is initiated prior to US presentation and the rise time is very rapid. Koekkoek et al reported significantly more rapid rise time (velocity analysis) in FX compared to healthy controls, but did not find impairment in onset or CR peak latency. As such, it is difficult to say whether or not our findings represent an adaptive or maladaptive response timing mechanism in FX. Nonetheless, our findings suggest that deviations from normality can be described best as disorganized, and our theoretical analysis suggests that such deviations are maladaptive.

The nature of the cerebellar dysfunction in FX and autism appears different between the two groups. Individuals with autism demonstrate significantly enhanced (i.e., rapid) acquisition while individuals with FX are impaired. However, published research investigating delay eyeblink conditioning in individuals with either autism or Fragile X syndrome have sampled two different age groups. Sears and colleagues (1994) and Arndt and colleagues (2008) each examined delay eyeblink conditioning in individuals with autism that were mostly younger than age 17 (Sears et al. included several participants as old as 22 years). Our sample included participants that were age 17 and older, with only four participants younger than age 30. While

it is tempting to conclude that delay eyeblink conditioning differentiates autism from Fragile X syndrome, the appropriate comparison of age-matched participants with either autism or Fragile X syndrome has not been conducted, precluding conclusive statements about group differences. However, there may be a link between the two disorders in that the CR timing, especially peak latency, appears to be similarly maladaptive for both FX and autism. Elucidation of this possible link between FX and autism requires additional studies of eyeblink conditioning that includes replication of the behavioral effects in addition to neurobiological analyses. Continued development of animal models of FX and autism should allow for such experiments.

Study 2

Delay eyeblink classical conditioning shows age related impairment beginning in middle age (Durkin, Prescott, Furchgott, Cantor & Powell, 1993; Solomon, Pomerleau, Bennet, James & Morse, 1989; Woodruff-Pak & Thompson, 1988). A potential neural mechanism for the aging-induced learning impairments may be related to neuro-degeneration of Purkinje neurons in the cerebellar cortex because the population of Purkinje neurons naturally declines with aging (Hall, Miller & Corsellis, 1975). Purkinje neurons are a natural component of the delay eyeblink classical conditioning circuitry (Thompson, 2005) as they activate neurons in the cerebellar deep nuclei and stimulate the plasticity of learning and long-term memory. Although Purkinje neurons are not necessary for the acquisition of associative information during delay eyeblink conditioning (Lavond & Steinmetz, 1989), they accelerate the rate of acquisition when they are present and functional (Woodruff-Pak, Cronholm & Sheffield, 1990). The documented Purkinje neuron degeneration that accompanies normal aging is also found in at least some cases of FX. For example, Sabaratnam (2000) found a reduced Purkinje neuron population in an individual case of FX at autopsy. However, this patient was elderly at the time of death and Sabaratnam did not report whether this reduced Purkinje neuron population was of similar magnitude as in normal healthy aging. As such, the reduced Purkinje population may be attributed to normal aging processes and may not represent a neuropathological characteristic of FX.

Delay eyeblink classical conditioning is retained over long intervals in healthy adults. Solomon and colleagues (Solomon, Flynn, Mirak, Bret, Coslov & Groccia, 1998) reported moderate retention of delay eyeblink conditioning after five years between assessments in a sample of adult participants. Normal aging affected eyeblink conditioning performance with older participants demonstrating significantly reduced retention, and elderly participants demonstrating very little retention, each compared to younger adult participants. In a similar study over a one-year retention interval, similar to the follow-up intervals examined in the study reported below, elderly human participants showed no change in response rate at the follow-up assessment, suggesting that elderly participants retain the ability to acquire and reacquire eyeblink conditioning (Woodruff-Pak et al. 1995).

Little is known about the cognitive aging of individuals with mental retardation. This second study was intended to identify whether individuals with FX retain delay eyeblink conditioning over 12-month retention intervals, and whether or not there are age differences within the FX population for the acquisition of eyeblink conditioning. Based on the available information, we expected FX subjects to display poor retention and reacquisition of delay eyeblink conditioning, and to demonstrate an effect of aging within the disordered population.

Method

Participants—Sixteen FX participants from Study 1 consented to participate in each of two successive 12-month follow-up sessions. Participants were divided into two age groups that were either older or younger than age 45 at the time of initial and follow-up testing. The younger

age group ($n=8$; 17-43 years) consisted of male ($n=6$) and female ($n=2$) participants. The older age group ($n=8$; 45-77 years) consisted entirely of male participants ($n=8$). Participants did not age out of the younger group as the follow-up data were collected over 24 months.

Apparatus—The eyeblink conditioning apparatus was the same as in Study 1.

Procedure—The testing procedure for eyeblink conditioning was the same as Study 1.

Analysis—All analyses were conducted using SPSS 14.0. Data from paired trials only were analyzed. Missing data values were replaced with the mean for that individual subject to preserve power. Violations of sphericity were handled as in Study 1. *Epsilon* is reported where necessary. Data from one subject could only be analyzed for percentage CRs. Table 2 displays descriptive statistics for EBC performance at each of the three testing sessions.

Results

Participants with FX showed a significant effect of aging on the reacquisition of delay eyeblink conditioning, although both age groups demonstrated significant plasticity with subsequent training. The younger age group demonstrated superior retention than the older age group based on a visual examination of the data (i.e., statistical methods were not employed for the analysis of retention). Analyses of each dependent measure collected during eyeblink conditioning are reported below in a 2 (Group: younger vs. Older) $\times$ 3 (Session) $\times$ 8 (Training Block) repeated measures ANOVA. Group is a between subjects variable, and Training Block and Session are within subject variables. The statistical analyses reported below reveal several main effects for Group, Training Block and Session, showing that participants in both age groups were able to increment their initial performance with subsequent training, and suggesting that older participants were impaired relative to the younger group of participants. In addition, results of the analyses revealed only non-significant differences between age groups for UR characteristics, including amplitude and peak latency. Such findings rule out potential confounds in the (dys)functionality of the motor pathway, and emphasizes age-related performance differences.

Age differences—FX participants were divided into groups of young FX (< 45 years) and old FX (> 45 years) participants for the analysis of age effects. The results of the ANOVA with CR percentage as the dependent variable show a significant main effect of Session $F(1,1.65) = 12.21, p < .001$ ($\epsilon = .825$), a significant main effect of Training Block $F(1,7) = 4.091, p = .001$, a significant Session $\times$ Age Group interaction $F(1,2) = 3.537, p = .043$, and a significant Session $\times$ Training Block interaction $F(1,14) = 1.838, p = .035$ (Figure 5). The significant Session $\times$ Age Group interaction indicates a different effect of age at each level of the Session variable, with no differences between age groups at Session 1, and superior performance of the younger FX group at Sessions 2 and 3 compared to the older FX group. The Session $\times$ Training Block interaction indicates a different rate of reacquisition during each of the follow-up sessions, although this effect is not related to the age of the participants. The main effect of Age Group and the three-way interaction for Group, Training Block and Session were non-significant.

Response Onset—The results of the ANOVA conducted to analyze follow-up performance of Response Onset latency indicate a significant Session $\times$ Age Group interaction, $F(1,2) = 3.854, p = .033$ (Figure 6). Pairwise comparisons reveal that response onset was not significantly different between age groups at Session 1, and was significantly different between age groups for Session 2 and Session 3, $ps = .004$ and $.001$, respectively. This interaction shows that younger participants demonstrated superior performance than older participants resulting from subsequent training. In addition, the analyses revealed a main effect of testing Session F

(1,2) = 11.123, $p < .001$, and a significant main effect of Training Block $F(1,7) = 3.253$, $p = .004$. The main effects for testing Training Block and Session are similar to the Group main effect observed in Study 1, in which Response Onset progresses from late (i.e., occurs after US presentation) motor response initiation to functional (occurs prior to US presentation) motor response initiation. Specifically, these two main effects show that Response Onset continued to adjust and normalized (for the younger FX group) with subsequent exposure to the eyeblink conditioning procedure.

CR Peak latency—The ANOVA with CR peak latency as the dependent variable revealed only non-significant differences for CR peak latency between Groups, as well as across any of the Sessions or Blocks. All two- and three-way interactions were also non-significant. Despite the statistically non-significant findings for CR peak latency, inspection of the data presented in Table 2 suggests that CR peak latency was disorganized throughout conditioning for both the younger and older FX groups. The younger FX participants produced a consistently prolonged peak latency compared to the older FX participants (the difference was non-significant), and the older FX participants continued to shift the timing of their peak CR in the maladaptive direction from Session 1 to Session 2. However, it is not possible to say whether or not these observations are abnormal characteristics of eyeblink conditioning in FX because longitudinal data from the healthy control participants is not available for statistical comparisons. Table 2 reports the CR peak latency for each group of participants for each of the training sessions. The data from the control participants appears only in the last column since they were not examined at the two follow-up training sessions. The data in the table shows that CR peak latency for the FX group was disorganized in terms of its modulation (failed to show consistent trajectory), and failed to normalize based on our observations of the matched control group from Study 1.

CR amplitude—The ANOVA with CR amplitude as the dependent variable yielded a significant interaction for Session x Training Block, $F(1,14) = 2.227$, $p = .008$, and Session x Age Group, $F(1,2) = 4.999$, $p = .014$ (Figure 7). The Session x Training Block interaction indicates that CR amplitude changed differently within a particular Session as compared to other Sessions. The Session x Age Group interaction indicates that older FX participants decreased the amplitude of their CRs from session 1 to session 2, whereas the younger FX participants increased the amplitude of their CRs from session 1 to sessions 2 and 3. In addition, there was a significant main effect of Session $F(1,2) = 4.701$, $p = .017$, and a significant main effect of Training Block $F(1,7) = 4.607$, $p < .001$.

UR characteristics—The ANOVA with UR amplitude as the dependent variable revealed a significant Session x Group interaction $F(1,2) = 4.56$, $p = .019$ indicating that the amplitude of the UR was different between younger and older age groups of FX participants depending on the testing session. UR amplitude increased significantly from Session 1 to Sessions 2 and 3 for the younger FX participants. In contrast, older FX participants demonstrated a reduction of UR amplitude from Session 1 to Sessions 2 and 3. We found a similar interaction effect for CR amplitude from session 1 to sessions 2 and 3, although it is not exactly the same pattern of change. Each of the other interactions and main effects were non-significant for UR amplitude.

The ANOVA with UR peak latency as the dependent variable revealed a significant main effect of Training Block $F(1,7) = 3.002$, $p = .007$, indicating that UR timing adjusted within a particular session as a result of exposure to the eyeblink conditioning procedure. This is a similar effect as reported in Study 1 above regarding the modification of reflex timing in response to repeated exposure to the contingencies corresponding to delay eyeblink classical conditioning, and indicates that the latency of the UR peak was significantly reduced as a result of direct experience with the US. The main effect of either Group or Session, and the

interactions were non-significant for UR peak latency. Table 2 presents this data along with other CR and UR characteristics for each group.

Discussion

The results of Study 2 show that the cerebellar dysfunction involved in FX is susceptible to aging with respect to retention and reacquisition of conditioned responding. Retention over each follow-up interval is indicated by similar performance in training block one as demonstrated in the final block of the previous testing session. FX participants demonstrated retention over the two 12-month intervals indicated by the increased percentage of CRs at the start of each follow-up session as compared to the initial acquisition session (Figure 5). It appears that younger FX participants demonstrated greater retention than older FX participants. The younger FX participants clearly demonstrated retention from Session 1 to Session 2 with respect to percentage of CRs. The effect is even more pronounced when percentage CRs at the end of Session 2 and beginning of Session 3 are examined. Older FX participants, on the other hand, did not demonstrate retention over the first 12-month follow-up interval, although there is evidence of retention over the second follow-up interval. These findings suggest that the initial impairment in percentage CRs observed in Study 1 is not a ceiling effect and is not attributable to a capacity-like restriction imposed by cerebellar dysfunction. Moreover, the results are similar to those obtained in rabbits with cerebellar cortex removed in that acquisition was significantly delayed but not abolished (Lavond & Steinmetz, 1989). These findings support the interpretation that cerebellar cortex is dysfunctional in FX, rather than cerebellar deep nuclei.

The incremented percentage of CRs observed during each reacquisition session suggests that the underlying cerebellar neuropathology may not preclude learning per se, but rather prolongs learning or impairs learning of temporal information that optimizes conditioned responding. Eyeblink classical conditioning includes learning of more than one simple CS/US association for adaptive functioning. In addition to learning the CS/US association, the timing between the CS and US must also be learned. Pathology that disrupts the learning of timing may necessarily disrupt the expression of CRs, and may implicate abnormal connectivity of the underlying network that can be overcome with additional exposure to paired training trials. For example, response onset latency appears to have normalized (i.e., resembles the performance of normal control group at Session 1) with subsequent training as can be seen in the reduction of response onset latency from Session 1 to subsequent sessions (see Table 2). In contrast, peak latency remained abnormally rapid throughout the three testing sessions and did not demonstrate adaptation toward onset of the US (again, see Table 2). These findings suggest that the mechanisms for acquiring the CS/US association and for timing the response onset of conditioned responses are highly interactive or interdependent, and that the mechanism that modulates peak latency is substantially less interactive or even independent of the other mechanisms involved in delay eyeblink classical conditioning. Moreover, FX and autism are each associated with declining adaptive functioning on longitudinal analyses of intelligence and cognitive behavior (Fisch, Simensen & Schroer, 2002). Such poor longitudinal adaptive functioning profiles in autism and FX, in conjunction with the poor adaptation of CR peak latency in our study of FX advocates peak latency as a good model of adaptive behavior that is separate from acquiring a simple associative contingency.

The effects of age observed in this study appear congruent with findings in normal aging where older participants have demonstrated impaired conditioned responding relative to younger participants (Durkin et al., 1993; Solomon et al., 1989; Woodruff-Pak & Thompson, 1988). The older FX group did not show impairment in initial acquisition relative to the younger FX group, but showed significant impairment in reacquisition compared to the younger FX group. Furthermore, the older FX group demonstrated disorganization of CR peak latency across

training sessions, whereas the younger FX group showed abnormal but not disorganized CR peak latency, and may be related to the significant interaction of age Group and Session for CR amplitude as well as CR timing variables.

Finally, UR amplitude was greater during the first session for older than younger participants, but the relationship reversed by the third training session with younger participants demonstrating greater UR amplitude. Previously we have reported significantly low UR amplitude in healthy older adults that is associated with smaller eye size in larger samples of older adults (Woodruff-Pak & Finkbiner, 1995; Woodruff-Pak & Thompson, 1988). In this sample of 8 older and 8 younger adults with FX, the cause for the shift in UR amplitude in the two age groups is unclear. Reflex facilitation (increased amplitude) and diminution (decreased amplitude) that are the result of associative processes may be attributable for the different effects of age on UR amplitude in the FX group. Flaten and Powell (1998) reported that conditioned reflex facilitation is differentially affected by age in younger and older adult humans. Given similar age effects in the FX group as in normal healthy participants for acquisition, it is possible that the same associative processes mediating facilitation and diminution of the UR in normal participants is preserved in our sample of FX participants. This is not in opposition to our suggestion that conditioning deficits reflect cerebellar cortical dysfunction, and in fact provides further support because Weisz and LoTurco (1988) have previously demonstrated reflex facilitation in rabbits with cerebellar cortex removed.

Our findings indicate that FX syndrome is characterized by poor delay eyeblink classical conditioning with respect to acquisition and several measures of response timing. The relationship between FX and autism remains unclear with this report. It seems that FX and autism are dissociable in terms of learning rate, but may be comparable in terms of response timing deficits. Regardless of the relationship between FX and autism for eyeblink classical conditioning, it appears that, although initially impaired for acquisition FX participants may show normalized levels of acquisition, and similar effects of aging as in the normal population. Whether these same effects are present in autism remains to be seen. In conclusion, individuals with FX demonstrate cerebellar dysfunction, and cerebellar cortical functioning may be associated with adaptive functioning in both FX and autism.

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Figure 1. Stimulus presentation timing scheme and sample waveform illustrating the eyeblink conditioning data that is analyzed. Onset (a) is observed during either response period and marks the time point representing the initial increase in amplitude necessary to observe a motor response. CR peak latency (b) and CR amplitude (c) are observed only during the CR period. UR peak latency (d) and UR amplitude (e) are observed only during the UR period.

Figure 2.

Mean percentage of CRs on paired CS/US trials for FX and Control groups. The acquisition curve for FX participants never intersects the curve for control participants illustrating the main effect of group detected by ANOVA. Error bars represent standard error of the mean.

Figure 3.

Mean response onset latency for FX and Control groups. The graph illustrates the main effect of group in which control participants adapted the response onset of their eyeblink response to occur prior to US onset (at 400 ms). The dashed line at 400 ms indicates stimulus-onset of the US. The FX participants demonstrated significantly delayed eyeblink response onset as revealed by ANOVA. Error bars represent standard error of the mean.

Figure 4.

Mean CR peak latency for FX and Control groups. FX and normal control participants shifted the peak latency of their conditioned eyeblink responses in different directions demonstrating the significant interaction between Training Block and Group. FX participants shift their CR peak latency as if the task were a reaction time task, rather than a controlled response timing task. Error bars represent standard error of the mean.

Figure 5.

Age differences for long-term retention and reacquisition of conditioned responding in FX participants at initial assessment and two successive 12-month follow-up sessions. Retention is evidenced by similar percentage of conditioned responses during each final and subsequent first training block during reacquisition. Younger FX participants demonstrated retention during both follow-up sessions, whereas older FX participants only demonstrated retention from the second to the third training sessions. Error bars represent the standard error of the mean.

Figure 6.

Age differences in response onset latency for Fragile X participants at each session. Both younger and older FX participants shifted conditioned response onset adaptively in all three training sessions. Younger FX participants showed normalized response onset during the second and third training sessions indicated by response onset occurrence prior to US onset (i.e., 400 ms). The dashed line in each panel indicates stimulus-onset of the US. Error bars represent standard error of the mean.

Figure 7.

CR Amplitude for older and younger Fragile X participants at each session. The significant interaction between Training Session and CR amplitude for younger and older FX participants is depicted in the change in amplitude from Session 1 through Session 3. In Session 1, younger participants demonstrated lower amplitude compared to older participants. In Sessions 2 and 3, younger participants demonstrated greater CR amplitude than older FX participants. Error bars indicate standard error of the mean.

Table 1

Subject Characteristics

	Age	Eye Size	BPM	Sex
Fragile X	Mean (SD)	Mean (SD)	Mean (SD)	Female (male)
under 45 (10)	27.23 (9.02)	8.60 (2.17)	12.4 (4.12)	3 (7)
45 and older (10)	64.62 (9.96)	7.90 (2.47)	29.2 (24.8)	0 (10)
Normal Control				
under 45 (10)	27.17 (9.26)	9.89 (1.83)	20.7 (11.83)	3 (7)
45 and older (10)	64.42 (10.24)	7.90 (1.29)	19.0 (11.6)	2 (8)

Note. Eye size is measured in millimeters. BPM = blinks per minute, or baseline blink rate.

Table 2

EBC Performance of FX Participants at Each Testing Session

	Session 1	Session 2	Session 3	Control
% CRs				
< 45	39.5 (12.9)	74.1 (15.7)	81.2 (9.0)	64.0 (19.5)
> 45	40.5 (9.7)	44.2 (12.1)	58.2 (7.2)	45.7 (25.7)
Response onset				
< 45	455.1 (35.9)	324.8 (37.4)	321.6 (25.9)	348.3 (50.1)
> 45	439.1 (35.1)	413.9 (28.0)	395.7 (24.2)	396.6 (83.5)
Peak Latency				
< 45	334.5 (7.9)	317.5 (27.2)	329.1 (21.6)	348.6 (29.1)
> 45	322.1 (17.5)	268.3 (44.1)	284.4 (47.2)	326.1 (29.5)
CR Amplitude				
< 45	2.5 (.9)	4.4 (1.4)	5.3 (.8)	3.93 (3.8)
> 45	3.25 (1.0)	2.5 (.8)	3.4 (.7)	1.63 (1.1)
UR Amplitude				
< 45	6.24 (1.58)	6.23 (2.32)	7.79 (5.94)	8.57 (3.1)
> 45	7.39 (4.16)	6.4 (2.98)	6.27 (3.1)	6.19 (1.9)
UR Peak				
< 45	472.2 (75.6)	483.4 (60.6)	466.2 (68.1)	473.7 (37.5)
> 45	481.3 (79.8)	503.8 (32.5)	476.4 (43.1)	501.9 (27.8)