EYEBLINK CONDITIONING IN PSYCHIATRIC CONDITIONS - STATE OF THE FIELD AND FUTURE DIRECTIONS

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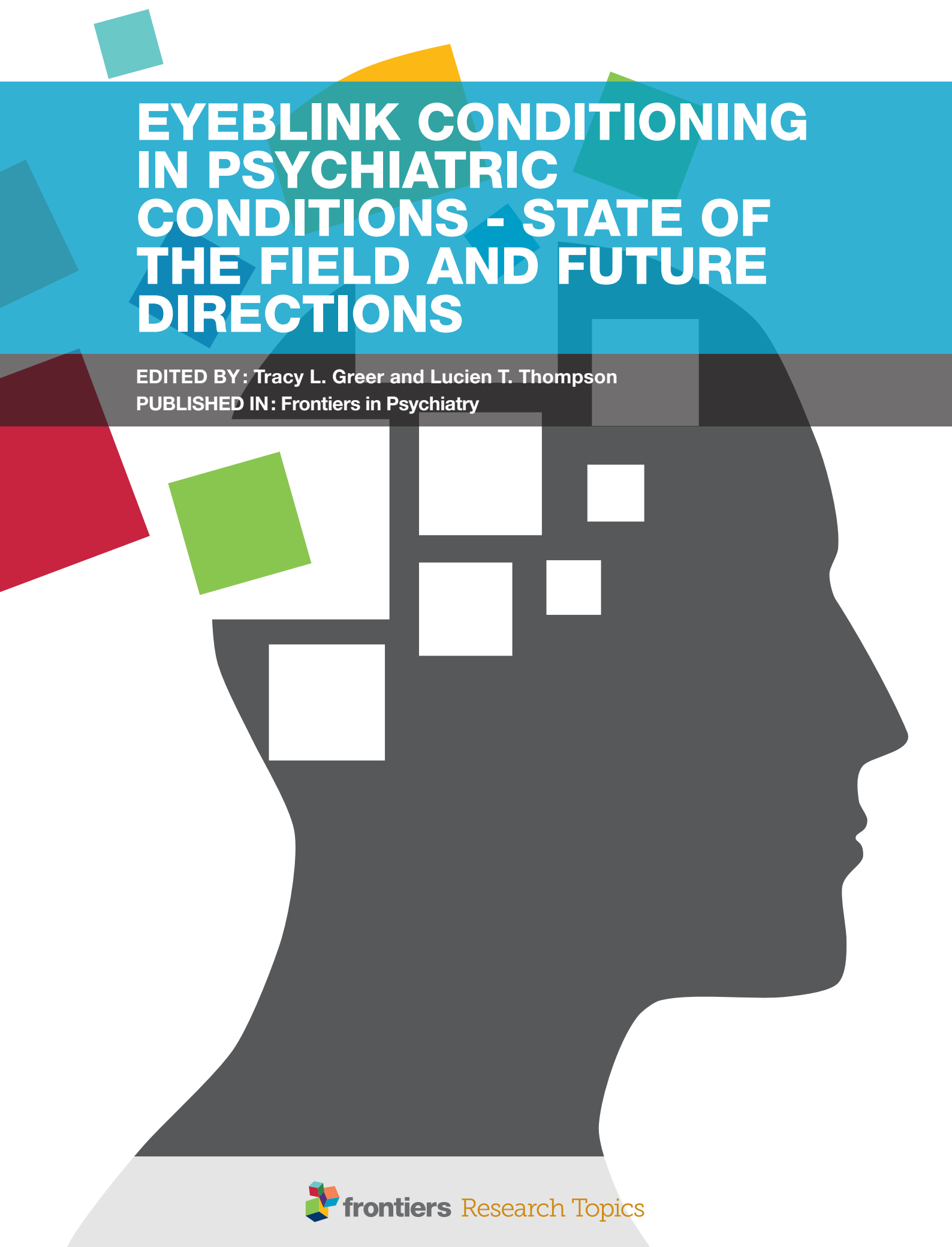

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EYEBLINK CONDITIONING IN PSYCHIATRIC CONDITIONS - STATE OF THE FIELD AND FUTURE DIRECTIONS

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Eyeblink classical conditioning (EBC) is a model paradigm for associative (also termed Pavlovian) learning, one of the simplest and best understood forms of learning and memory. Because EBC paradigms are readily adapted across species, the neural substrates of EBC have been well characterized, and include but are not limited to the cerebellum and anterior interpositus nucleus, the hippocampus, and prefrontal cortices. The ability to collect EBC data across many different species (i.e. including but not limited to humans) also has the distinct advantage of facilitating translational research, and therefore may be of particular benefit to elucidate mechanistic changes associated with a wide variety of psychiatric disorders.

In fact, EBC paradigms have been employed to assess individuals with a wide range of neurological deficits (including Korsakoff's amnesia, Alzheimer's disease as well as normal aging, dyslexia, inflammatory tremor, dystonia, and multiple sclerosis) and psychiatric disorders (including major depressive disorder, anxiety disorders, schizophrenia, autism, and alcohol use/addiction disorders). Individuals with these disorders exhibit differential impairments across different EBC task types (e.g., delay vs. trace EBC), with some showing impairment in one but not the other task and some showing impairments in both; across learning stage (e.g., acquisition, discrimination, or extinction), and across response variables (e.g., magnitude and timing of the conditioned eyeblink motor response, modality of the conditioned stimulus). Evaluating specific individual differences in the context of variable brain pathology should aid characterization and refinement of our understanding of complex neuropsychiatric disorders.

The field of psychiatry has seen a transition from more traditional use of symptom clusters to define psychiatric disorders with subsequent examination of associated behaviors and traits, to the use of physiological and behavioral indicators to characterize individuals with respect to various psychological domains [in line with the National Institute of Mental Health Research Domain Criteria (RDoC) initiative]. This approach employs a neuroscience-based framework to assess the pathophysiology of chronic mental illnesses. Behavioral and cognitive processes are critical domains of interest in evaluating potential maladaptive patterns that may be indicative of specific psychopathologies. Furthermore, the rapid development of technological advances that allow for more detailed examination (e.g., EEG, MEG, MRI, fMRI, infrared

imaging) and manipulation (e.g. transcranial magnetic and direct current stimulation) of brain functions should enhance our ability to better characterize EBC performance and its utility in characterizing aspects of particular neuropathologies.

Substantial research evidence exists for the value of EBC paradigms to inform our understanding of the pathophysiologies underlying a wide variety of neurological and psychiatric disorders. Despite these findings, this readily implemented classic cognitive-behavioral paradigm is relatively underutilized in clinical settings. This e-book highlights recent convergence of clinical and research efforts in this area and aims to promote a resurgent interest in eyeblink classical conditioning, and to emphasize the potential for future translational and diagnostic applications of EBC in combination with other techniques to strengthen our understanding of alterations in brain function manifested in behaviors characteristic of specific psychopathologies.

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EDITORIAL
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Editorial: Eyeblink Classical Conditioning in Psychiatric Conditions: Novel Uses for a Classic Paradigm

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Keywords: schizophrenia, alcohol abuse, hippocampus, frontal cortex, cerebellum, post-traumatic stress disorder, autism, depression

Editorial on the Research Topic

Eyeblink Classical Conditioning in Psychiatric Conditions: Novel Uses for a Classic Paradigm

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As one of the most basic forms of associative learning, eyeblink conditioning (EBC) is a model paradigm with unique utility in the assessment of complex behavioral disorders, including psychiatric disorders. Two major EBC paradigms utilized with human subjects are delay EBC [in which a conditioned stimulus (CS; e.g., an auditory tone) co-terminates with an unconditioned stimulus (US; e.g., a corneal airpuff)] and trace EBC [in which CS presentation is followed after a silent interstimulus interval (termed the "trace" interval by Pavlov) by the US, with no CS-US overlap in time]. The neural substrates of EBC in these paradigms are well delineated and include the cerebellum and anterior interpositus nucleus, the hippocampus, and prefrontal cortex. Variability in acquisition, discrimination, timing, sensitization, and/or extinction of classically conditioned eyeblink responses provides insight into the behavioral and neurobiological characteristics of a variety of psychiatric and neurological disorders.

In this Research Topic, Kent et al. review the existing literature on EBC studies in schizophrenia, perhaps the most studied psychiatric diagnostic group with respect to EBC to date. In their review of 15 studies, they report that decreased percent CRs (impaired learning) is the most robust and replicated finding in this population, with equivocal findings associated with CR timing. Importantly, differences between schizophrenic and healthy controls in percent CRs appear to be reflective of cerebellar abnormalities—supported by neuroimaging data and data from first-degree relatives—rather than confounding issues such as medication use. However, the authors highlight the importance of methodological consistency across EBC paradigms for accurate interpretation and synthesis of data across studies, an issue critical in the evaluation of any behavioral measure. Bolbecker et al. report data on delay EBC in schizophrenia using an analytic approach—hierarchical liner modeling—that has the advantages of less restrictive assumptions and inclusion of unequal variances in comparison to more traditionally employed analytic approaches, such as repeated measures analysis of variance. The authors did not find differences between schizophrenic patients and age-matched healthy controls in learning rate, but did observe group differences in time to maximal learning level and plateau of learning response, with schizophrenic patients exhibiting saturation in learning earlier and at a lower level in comparison to healthy controls.

Cheng et al. provide summaries of both animal and human work describing the impact of alcohol use disorders in adults and fetal alcohol syndrome in children. EBC studies in these populations have illuminated structural and functional differences in both the mature and developing cerebellum, as well as learning differences in these diagnostic groups when compared to healthy controls.

Weiss and Disterhoft emphasize the utility of rabbit EBC for assessing cerebro-cerebellar functional connectivity. The role of hippocampus, fronto-temporal and cerebellar cortices, and of cerebellar deep nuclei has been well characterized in a wide variety of normal states as well as dysfunctional psychiatric conditions. Their review concentrates on models of schizophrenia and Alzheimer's dementia, but also points out applications for normal aging, Parkinson's disease, progressive supranuclear palsy, alcoholism, and post-traumatic stress disorder (PTSD). Indeed, the rabbit EBC model was developed in parallel with assessment of human EBC in a series of studies from the laboratories of Gormezano, his students, and colleagues, which have allowed mechanistic, physiological, and pharmacological assessments to be carried out in depth.

Schreurs and Burhans detail development of a preclinical EBC extinction model with potential utility for treatment of inappropriate conditioned behaviors and hyperarousal in individuals suffering from PTSD, whether combat veterans or approximately 5–25% of the general populace who experience severe anxiety, sleeplessness, hypervigilance, and/or flashbacks after trauma constitute a group at extreme risk for suicide. Their exposure paradigm uses conditioning-specific reflex modification and attenuated intensity US presentations to enhance the likely clinical utility for human applications.

Janke et al. discuss dysregulation of brain-derived neurotrophic factor (BDNF) in anxiety disorders, including impaired behavioral inhibition temperament (BI). They describe facilitated delay EBC in both human and animal subjects exhibiting BI. In their animal model after EBC, increases in mRNA for hippocampal BDNF were observed in both the dentate gyrus and CA3 regions, along with upregulation of TrkB receptors and downstream activity-related cytoskeletal protein (Arc) in the hippocampus, but these increases were blunted in the strain showing faster acquisition and hyper-sensitivity to both CS and US. Hippocampal BDNF administration reversed the behavioral sensitization, suggesting treatment strategies that enhance that hippocampal BDNF may be effective for a variety of anxiety disorders.

Welsh and Oristaglio provide a secondary analysis of children with autism spectrum disorder (ASD) who underwent both delay and trace EBC. They grouped children based on their diagnosis into either an autistic disorder group or an Asperger's syndrome or Pervasive Developmental Disorder (Asp/PDD) group. Neither ASD group showed differences in CR acquisition compared to an age- and IQ-matched group of typically developing (TD) children. However, the groups differed with respect to CR timing alterations, with the Asp/PDD group showing delayed CR onset and peak latencies during trace conditioning that were not observed in the ASD or TD groups. These data may illustrate differences in the underlying biology of these two diagnostic groups that are expressed as behavioral differences. Although the authors indicate that these differences must be further tested in a larger trial, these data are representative of the types of approaches that are supported by the NIMH's Research Domain Criterion (RDoc) initiative that aims to use a variety of approaches, including behavioral phenotyping, to help our field better distinguish brain dysfunction among patients with psychiatric illnesses.

Similarly, Parker describes the potential for timing tasks to aid in our understanding of cognitive impairment across diagnostic groups, again in line with the RDoC approach. She asserts that tasks that involve temporal processing, such as EBC, can reflect connectivity between the cerebellum and frontal cortex, and in particular, hypothesizes that the medial frontal cortex plays a critical role in cognitive processing that occurs in timing tasks. This reciprocal relationship between cerebellar and frontal cortical regions is an important area of further investigation.

Cicchese and Berry also focus on timing and the critical role of theta (the 3–7 Hz EEG bandwidth prominent in medial temporal lobe) in many forms of learning and memory. They review non-theta contingent EBC, demonstrating its importance as a model system for characterizing neurobiological dysfunction in severe cognitive disorders, including schizophrenia, major depression, and Alzheimer's disease. From their animal studies and an extensive review of the human literature, they argue that theta rhythms serve to coordinate timing and synchrony of activity in widely distributed brain systems critical for acquiring, consolidating, and retrieving memories, both for complex cognitive sequences and for relatively low-level tasks such as classical EBC.

The studies in this Research Topic highlight the utility of EBC in assessing the integrity of cerebellar and medial temporal lobe function in both normal and pathological states and support wider uses of this behavioral paradigm across a multitude of psychiatric disorders. The editors' earlier work on impaired trace EBC in depressed individuals (1) illustrates qualitative diagnostic potential for this simple associative learning paradigm in future clinical practice. Increased familiarity of clinical practitioners with the straightforward methodology required, and adoption of standards for EBC testing and adjunct assessments of clinical characteristics of populations studied, should still further increase the use of this classical conditioning paradigm in diagnostic and treatment settings.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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1. Greer TL, Trivedi MH, Thompson LT. Impaired delay and trace eyeblink conditioning performance in major depressive disorder. *J Affect Disord* (2005) 86:235–45. doi:10.1016/j.jad.2005.02.006

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REVIEW
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Eyeblink conditioning and novel object recognition in the rabbit: Behavioral paradigms for assaying psychiatric diseases

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Analysis of data collected from behavioral paradigms has provided important information for understanding the etiology and progression of diseases that involve neural regions mediating abnormal behavior. The trace eyeblink conditioning (EBC) paradigm is particularly suited to examine cerebro-cerebellar interactions since the paradigm requires the cerebellum, forebrain, and awareness of the stimulus contingencies. Impairments in acquiring EBC have been noted in several neuropsychiatric conditions, including schizophrenia, Alzheimer's disease (AD), progressive supranuclear palsy, and post-traumatic stress disorder. Although several species have been used to examine EBC, the rabbit is unique in its tolerance for restraint, which facilitates imaging, its relatively large skull that facilitates chronic neuronal recordings, a genetic sequence for amyloid that is identical to humans which makes it a valuable model to study AD, and in contrast to rodents, it has a striatum that is differentiated into a caudate and a putamen that facilitates analysis of diseases involving the striatum. This review focuses on EBC during schizophrenia and AD since impairments in cerebro-cerebellar connections have been hypothesized to lead to a cognitive dysmetria. We also relate EBC to conditioned avoidance responses that are more often examined for effects of antipsychotic medications, and we propose that an analysis of novel object recognition (NOR) may add to our understanding of how the underlying neural circuitry has changed during disease states. We propose that the EBC and NOR paradigms will help to determine which therapeutics are effective for treating the cognitive aspects of schizophrenia and AD, and that neuroimaging may reveal biomarkers of the diseases and help to evaluate potential therapeutics. The rabbit, thus, provides an important translational system for studying neural mechanisms mediating maladaptive behaviors that underlie some psychiatric diseases, especially cognitive impairments associated with schizophrenia and AD, and object recognition provides a simple test of memory that can corroborate the results of EBC.

Keywords: Alzheimer's disease, cerebellum, cognitive dysmetria, hippocampus, prefrontal cortex, schizophrenia

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Neuropsychiatric diseases are a significant worldwide health issue. Analysis of data collected from behavioral paradigms has provided important information for understanding the etiology, and progression of diseases that involve neural regions mediating abnormal behavior. Behavioral paradigms also provide systems for testing potential treatments and therapeutics. Eyeblink conditioning (EBC)

is one such behavioral paradigm. This paradigm pairs a neutral conditioning stimulus (CS), e.g., a brief tone, flash of light, or vibration of whiskers with a mildly aversive stimulus to the eye or surrounding area in order to evoke a conditioned blink response. Subjects become conditioned after several pairings of the stimuli such that a blink is evoked in response to the CS and prior to the onset of the aversive unconditioned stimulus (US). Importantly, control experiments indicate that the learning is associative in nature, i.e., blinks do not tend to occur to the CS when it is presented in a random unpaired schedule with the US.

Learning occurs most quickly when onset of the US is delayed from the onset of the CS by approximately 250 ms, and when the CS and US overlap and coterminate in time [longer interstimulus intervals (ISIs) are optimal for human subjects]. The 250 ms ISI is the shortest interval tested in the rabbit by Schneiderman and Gormezano (1). Several studies have found that generation of a conditioned response (CR), a blink that occurs prior to the onset of the US and which protects the eye from the noxious stimulus, requires the thalamus, cerebellum, and afferent inputs from the brainstem to the cerebellum (2–5). However, learning the task is more difficult when a stimulus-free interval separates the two stimuli during a trial, i.e., more trials are required before CRs are exhibited (6). The simple addition of this stimulus-free "trace" interval between the two stimuli increases the memory demand of the task, recruits forebrain areas that would otherwise not be required for the task, and importantly requires awareness that the CS predicts the occurrence of the aversive stimulus [as reported by human subjects (7, 8)]. The requirement for awareness makes trace EBC a useful paradigm to investigate the cognitive nature of cerebellar function as proposed by Leiner et al. (9, 10), and abnormalities in the cerebro-cerebellar circuitry that mediates awareness likely involves the circuitry that makes EBC sensitive to neuropsychiatric disease.

The distinction between the neural requirements for the delay and trace versions of the EBC paradigm allows behavioral testing to dissociate forebrain-dependent cognitive effects from a more basic sensorimotor integration mediated by the brainstem/cerebellar/thalamic systems. Although EBC has been used most often to study neural mechanisms mediating learning and memory in healthy adults, the dissociation between forebrain and cerebellar/brainstem effects is useful in helping to characterize the effects of a disease state, and the effects of a potential treatment.

Several reports indicate that EBC can be used to detect impairments in neuropsychiatric diseases, such as schizophrenia (11–14), Alzheimer's disease [AD (15–17)], progressive supranuclear palsy [PSP (18)], and post-traumatic stress disorder [PTSD (19)] EBC is significantly impaired by AD, relative to age-matched control subjects (15, 17). There is the one report of EBC in patients with PSP, which indicates a severe impairment in acquiring EBC with trace intervals of 0, 300, or 600 ms (18); those authors concluded that the deficit was likely due to neuropathological changes in the cerebellar nuclei since other pathologies overlap with those of Parkinson's disease (PD), which does not impair acquisition of EBC (20). The effects of PTSD on EBC are discussed by Schreurs and Burhans elsewhere in this volume (19). The EBC paradigm also reveals age-related learning impairments in humans (21–23), rabbits (24), and rats (25–28). Overall, the EBC paradigm is quite translational in nature. The phases of behavioral acquisition are similar between human and non-human subjects (although scaled differently) and many of the same stimuli and stimulus delivery systems can be used with both types of subjects (29). Much of our understanding of the neural networks mediating this conditioning comes from *in vivo* recordings from single neurons and multiunit activity in different brain regions during the task (30–35), and from permanent and temporary lesions of regions suspected to be involved in the task (3, 4, 33, 36–39).

Although this review focuses on the benefits of using the rabbit as the experimental subject, considerable advances have been made by using the mouse as a subject for EBC and deserve mention, especially for manipulations of the cerebellum and different transmitter systems. An understanding of the neurotransmitters and receptors involved in conditioning and cognition has been facilitated by using knockout and transgenic mice, e.g., elimination of monoamine oxidase isoenzymes A and B increases levels of monoamines, including serotonin (40) and resulted in abnormally enhanced acquisition rates of delay EBC, elevated levels of hippocampal long-term potentiation, decreased ratio levels of NMDA receptor subunits NR2A and NR2B in prefrontal cortex (PFC) [increased ratio levels in hippocampus (41)] and the adenosine receptor has been shown to be important in both acquisition of EBC and the development of LTP (42). These studies are of interest given the involvement of NMDA receptors and serotonin in schizophrenia (43–45).

In terms of the cerebellum, elimination of cannabinoid receptor 1 (CB1), which is highly expressed in cerebellum, or mutations of the glutamate receptor mGluR1 (46) or subunit delta2 which affects cerebellar cortex was found to significantly impair delay conditioning, but not trace conditioning [(47, 48), see Ref. (49) for a discussion of this result], and elimination of calcium/calmodulin-dependent protein kinase type IV (CaMKIV), which is expressed in cerebellar granule and nuclear cells, impaired long-term retention of delay conditioned blinks (50). These studies are of interest given the role of cerebellar–cortical interactions with schizophrenia (51).

In terms of AD, the insertion of genes related to AD have been shown to accelerate impairments in mice acquiring EBC (52, 53) and reduce the volume of their hippocampus, as measured with MRI (54). However, the genetic sequence for amyloid in the mouse is different than the sequence found in human amyloid. This adds the complication of foreign DNA in the host. By contrast, the rabbit sequence for amyloid is identical to the sequence in humans (55) and should minimize that complication. Lastly, learning specific changes in the cortical representation of the CS for whisker-signaled conditioning have been described (56) and provide a substrate for experimental manipulation.

A circuit diagram of relevant brain regions involved in trace and delay EBC is shown in **Figure 1**. Note that five modules have been identified: cerebellum, PFC, limbic-medial temporal, sensory cortex, and basal ganglia. The thalamic nuclei connecting the different modules are also shown (the anterior thalamus (AT) includes anterior dorsal, anterior ventral, and anterior medial). The circuit shows the flow of information representing the conditioning stimuli through the cerebellum, the forebrain, and back to the cerebellum by way of the pontine nuclei. The disruption of any

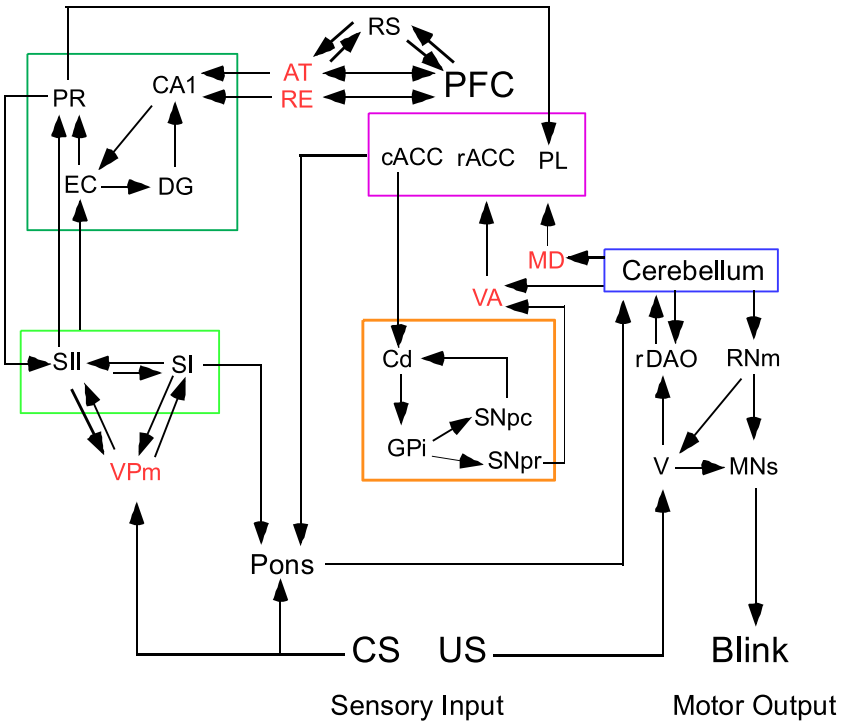

FIGURE 1 | Trace eyeblink conditioning requires forebrain input to a cerebellar circuit that mediates conditioning. Four forebrain circuits that interact via thalamic nuclei (shown in red text). The limbic, medial temporal circuit is outlined in dark green and is sensitive the effects of aging. The limbic forebrain circuit is outlined in magenta and is affected during schizophrenia. The basal ganglia circuit is outlined in tan and is affected during supranuclear palsy. The sensory circuit is outlined in light green (the somatosensory system is shown in this example). The cerebellar circuit is shown to the far right. The conditioning stimulus (CS) is conveyed to the cerebellum via mossy fibers originating in the pontine nuclei; the unconditioned stimulus (US) is conveyed via climbing fibers from the inferior olive. AT, anterior thalamus; cACC, caudal anterior cortex; Cd, caudate; DG, dentate gyrus; EC, entorhinal cortex; GPi, globus pallidus internal; MD, medial dorsal thalamus; MNs, motor neurons (facial and accessory abducens for blink conditioning); PFC, prefrontal cortex; PL, prelimbic cortex; PR, perirhinal cortex; rACC, rostral anterior cingulate cortex; rDAO, rostral dorsal accessory olive; RE, nucleus reuniens; RNm, magnocellular red nucleus; RS, retrosplenial cortex; SI, primary sensory cortex; SII, secondary sensory cortex; V, trigeminal nucleus; VA, ventral anterior thalamus; VPm, ventral posterior medial cortex; SNpc, Substantia Nigra pars compacta; SNpr, Substantia Nigra pars reticulata.

of the pathways or nuclei will lead to maladaptive responses to the stimuli regulating learned behaviors and to disrupted executive functions due to changes in the PFC.

The cerebellum is a necessary component for acquisition and expression of conditioned blink responses (2, 57). It is one synapse removed from the motor neurons that control the CR and importantly, it provides feedback to the frontal cortex via the thalamus (58–60). Removal of this input may contribute to a cognitive dysmetria and symptoms of schizophrenia (51). Destruction of the cerebellar nuclei (the sole output of the cerebellum) eliminates acquisition and expression of CRs, but leaves intact the unconditioned, reflexive eyeblink to noxious stimuli. The PFC is required for acquisition of EBC when the task is cognitively demanding as in trace conditioning or when the CS is relatively mild and requires attention for detection, even during delay conditioning (61). Acquisition of trace EBC requires the caudal anterior cingulate portion of the PFC [cACC (36)], and long-term retention involves the prelimbic (PL) portion (35). Lesions of the hippocampus result in non-adaptive short-latency CRs or with enough damage the animal is unable to acquire CRs (6, 62). Lesions of SI prior to whisker-signaled trace EBC prevent acquisition of CRs, but similar lesions made after consolidation has been allowed to occur for 30 days does not abolish CRs. We suggest that CS information is relayed into the hippocampal formation via the secondary sensory cortical system after consolidation has occurred. The role of the striatum was examined because of cognitive deficits associated with PD (63–66). Lesions of the caudate nucleus prevent acquisition of CRs (33) and similar lesions made after acquisition prevent any further improvement in expression of the CR (67).

Although most recording and lesion techniques are invasive and not appropriate to study in humans, functional magnetic resonance imaging can be done in both human and non-human animal subjects during and after learning (68–70). Blink conditioning thus provides an important translational tool for studying the neural mechanisms mediating maladaptive behaviors that underlie some psychiatric diseases. Here, we review some of the work that has been done with schizophrenia as a prototypical psychiatric disease and suggest ways in which the paradigm may be used to test potential therapeutics.

Other neuropsychiatric diseases have also been examined with EBC, e.g., AD, PSP, PD, and PTSD. Briefly, AD significantly

impairs acquisition relative to age-matched control subjects (15), acquisition is normal in patients with PD but impaired in patients with PSP (17, 18), and PTSD has effects (especially on the unconditioned response) as discussed elsewhere in this issue by Schreurs and Burhans (19).

Schizophrenia

Schizophrenia, a neuropsychiatric syndrome that includes symptoms of hallucinations, delusions, and extremely disordered thinking affects approximately 1% of the population. Behavioral abnormalities related to schizophrenia usually appear in the late teens and causes a life-long disability. Much evidence suggests that schizophrenia is a neuro-developmental disorder affecting connections between the cerebellum and PFC, which leads to a cognitive dysmetria (51, 71). More recently, an analysis of cerebellar gray matter using a modern unbiased morphometry approach, rather than whole-brain voxel based morphometry, found that gray matter volumes in Crus I/II were significantly reduced among patients, and the reduction correlated with tests measuring thought disorders and executive functioning (72).

Schizophrenia should affect both trace and delay conditioning since the cerebellum is required for both the delay and trace versions of the paradigm (73), even though the PFC is not required for the less demanding delay paradigm when salient stimuli are used (36). The connections between the cerebellum and PFC have been studied in non-human primates by Peter Strick and his group (58, 60). They found that neuronal loops connect the dorsolateral PFC and the cerebellum, and that the dentate cerebellar output nucleus of the loop is active during cognitive processing, as measured with functional magnetic resonance imaging [fMRI; (74)]. Cerebellar activation, as measured during fMRI based experiments has yielded mixed results, but a meta-analysis of more than 200 studies (75) found that approximately 40% of reports included individuals with schizophrenia and cerebellar hypoactivation was found in approximately two-thirds of those patients, mostly during tasks testing cognition and executive functions.

We have also used fMRI to measure the blood oxygen level-dependent (BOLD) response from the cerebellum in rabbits conditioned to evoke eyeblinks. We demonstrated learning-related decreases in the cerebellar cortex and learning-related increases in the deep cerebellar nuclei (68). We have also shown with multiple single-neuron tetrode recordings that neurons in the caudal anterior cingulate region (cACC) of the PFC exhibit conditioning specific increases in activity early in the trial sequence that appear to reflect a signal for attention to sensory stimuli. Conversely, neurons in the prelimbic area exhibit robust neuronal activation in response to the CS during tests for retention of remotely acquired EBC, i.e., the rabbits were trained to criterion and then left in their home cages for 30 days (35). Although the exact homolog of the primate dorsolateral PFC is difficult to establish in lower species, the activity pattern we reported for neurons in the prelimbic cortex appears to be a signal that reflects retrieval of the memory for how to respond appropriately to the conditioned stimulus, especially since the activity pattern was not evident during the relatively few trials when CRs were not expressed.

Interactions between the cerebellum and forebrain use relatively long axonal tracts and information processing within the PFC (and elsewhere), and is dependent on the proper functioning of the neurons and interneurons within the region. Abnormalities in GABAergic neurons have been proposed to contribute to the symptoms of schizophrenia. Changes in the inhibitory neurons of the PFC, especially of the dorsolateral PFC, have been reviewed by Lewis et al. (76). They proposed that GABAergic neurons in schizophrenic patients have defects in signaling pathways such that expression of the messenger RNA for GAD67, an enzyme involved in the synthesis of GABA, is reduced and postsynaptic $GABA_A$ receptors are upregulated. These deficits in the PFC could account for the disturbances in working memory (43), possibly due to a hypoglutamatergic state since antagonists of NMDA receptors, e.g., ketamine or phencyclidine (PCP), induce hallucinations similar to those observed in people with schizophrenia, and administration of PCP prevents acquisition of trace, but not delay EBC in rabbits (77).

Myelination defects in the cerebellar–prefrontal tracts are also thought to be involved in schizophrenia and have been hypothesized to lead to a functional disconnection between the two regions and a cognitive dysmetria (71). This disconnection could account for the hypoactivation found in the PFC of schizophrenic patients during imaging studies (78). A study of intrinsic connectivity between the cerebellum and the rest of the brain in schizophrenic patients, their siblings, and controls supports the hypothesis of a functional disconnection (79). This study found that patients had significantly impaired connectivity between the cerebellum and forebrain regions, including the hippocampus, thalamus, and middle cingulate gyrus (79). Each of these brain regions, and the cerebellum, are critically involved in mediating trace EBC (3, 4, 6, 31, 36, 38, 79–81).

Schizophrenia and Blink Conditioning Studies

The literature discussed so far suggest that patients with schizophrenia should have impaired acquisition of both delay and trace EBC because of defects in the cerebellum and thalamus/PFC, respectively. However, initial studies of EBC in patients with schizophrenia yielded mixed results. A review by Lubow (82) concluded that the inconsistencies in results were likely due to differences in the medication history of the patients. Lubow's conclusion was that the comparison between controls and patients that have or have not been medicated needs to be done in the same study to determine if symptoms are due to the disease *per se*, or due to interactions with medications. Those types of studies have been done (with delay conditioning) since the review by Lubow (11, 13, 82, 83); all of these more recent studies found that the groups with schizophrenia had impaired performance as compared to matched control subjects. The report by Coesmans et al. (83) is noteworthy in that the patients were recently diagnosed with schizophrenia (which limited the effects of medication), and no consistent effect of medication was found on conditioning (clozapine vs. haloperidol), i.e., all patient groups were impaired relative to control subjects. A report by Bolbecker (84) is also noteworthy in that a cerebellar dependence was

demonstrated by the subcutaneous administration of secretin (an agonist of group B G-protein coupled receptors), which acts as a retrograde messenger and neuromodulator on cerebellar basket and Purkinje cells. The compound significantly improved delay EBC in medically stable schizophrenic patients, as compared to patients that received a placebo control (controls showed no significant improvement in performance across trial blocks). These data suggest that it is also necessary for the cerebellar cortex to function properly in order for conditioning to occur properly.

Although early studies examining conditioning in schizophrenic patients are difficult to interpret due to differences in medication history, two of the studies are of particular interest in that they measured the level of arousal during the conditioning session. Mednick (85) found that the percentage of CRs correlated with the subjects' skin potentials, which indicated that the subjects were more aroused. Spain (86) found a similar result, although that experiment may have been confounded by an instruction to press a response key at the termination of the CS (1000 ms CS, 500 ms ISI, 160 ms US). These results are of interest due to interactions with executive functions of the PFC and the sensitivity of the PFC to the modality of the US used during trace conditioning studies. Oswald et al. (87) found that lesions of the PFC [anterior cingulate region (24)] impaired acquisition much more when the US was a puff of air to the cornea as compared to a shock to the periorbital region. The shock US appears to be able to compensate for deficits that might otherwise occur when a less salient stimulus is used.

The effects of arousal on responses to stimuli may be mediated by interactions of the PFC and hippocampal system via thalamic nuclei, including the anterior thalamic nuclei. This system has been examined with spatial memory tasks (88), but little is known about the system during EBC. We suggest that the greater arousal state of schizophrenic patients may be due to impaired circuitry in the prefrontal–thalamic–hippocampal system, which is then less able to respond properly to stimuli that are behaviorally important.

Effects of Neurotransmitters and Drugs on Eyeblink Conditioning

The EBC paradigm is an excellent model system to study behavioral pharmacology. Several drugs and transmitter systems have been examined using EBC. Acetylcholine (ACh) was one of the first neurotransmitters examined for effects on EBC. Given the involvement of the hippocampus in EBC (89), and the widespread role of ACh, Solomon et al. (90) examined the effects of scopolamine, a cholinergic, muscarinic antagonist on EBC in the rabbit. They found that systemically administered scopolamine severely impaired acquisition of delay EBC, but not when tested in rabbits that had their hippocampus ablated prior to the experiment. This demonstrates that a malfunctioning hippocampus (due to low ACh) is more of a detriment to learning than having no hippocampus at all, and suggests that abnormal neuronal transmission through the hippocampal system is likely to contribute to the cognitive impairments associated with schizophrenia.

Haloperidol was the next major drug examined for effects on EBC. This antipsychotic medication blocks dopamine (D2), alpha 1, and 5-HT2 (serotonin) receptors, among others, and has been shown to impair the acquisition rate for EBC (91). The impairment appeared to be due to an elevation in the threshold for an auditory CS to elicit CRs and suggests that the drug may be affecting attentional mechanisms and neuronal processing of the auditory cue since the effect was present when a 75 or 85-dB tone was used, but not when a 95-dB tone was used as a CS (92, 93).

The effects of serotonergic receptors on cognition, psychoses, and EBC deserve further review. An analysis of their effects on EBC has been investigated by John Harvey (94). He and his colleagues manipulated serotonergic receptors with agonists and antagonists during EBC and found that lysergic acid diethylamide (LSD) facilitated acquisition of CRs due to enhanced activation of the 2A/2C receptors unless the receptors were blocked by an antagonist, e.g., by Ritanserin (95). Since a 5HT1A agonist (8-OH-DPAT) had no effect, the effects of LSD are likely to be acting through the 2A/2C receptors rather than the 1A receptor. Harvey et al. (96) also increased the density of 5HT2A receptors in the frontal cortex by injecting MDL11,939 (a potent 5-HT2A antagonist) daily for 8 days prior to starting conditioning trials. The results indicated that the treated rabbits acquired CRs significantly faster than did rabbits given the vehicle control, and rabbits given the drug and explicitly unpaired stimuli exhibited <5% of trials with either spontaneous blinks or pseudo-CRs, suggesting that the drug was not acting on non-associative process.

Lastly, the *N*-methyl-D-aspartate (NMDA) receptor is the major excitatory receptor in the brain and is altered during learning and memory to facilitate ionic flow through its channel. Antagonists of the NMDA receptor (e.g., PCP, MK-801) are known to induce psychosis and have been found to impair EBC significantly in a dose-dependent manner in rabbits (77). Conversely, GLYX-13 (a novel NMDAR glycine-site functional partial agonist) facilitates acquisition of EBC in young and aging rats (27, 97).

Other Behavioral Paradigms for Evaluating Schizophrenia

We have focused our discussion on EBC as a behavioral paradigm to evaluate the effects of the schizophrenic condition. This behavior could be considered as a conditioned avoidance response (CAR), the type of response that has classically been observed to evaluate the effectiveness of antipsychotic medications, i.e., suppression of the CAR (43). However, CAR paradigms typically evaluate responses that occur over the course of several seconds, as in moving away from a region to avoid a foot-shock. By contrast, movements related to EBC occur over the course of a fraction of 1 s. Regardless, both types of paradigms involve a CAR and should produce similar results. An examination of EBC under conditions that model the schizophrenic condition might allow a test of this hypothesis.

As alluded to earlier, EBC works so well with rabbits because it requires minimal behavioral output from the rabbit, and rabbits do not express much spontaneous behavior that might otherwise interfere with the behavior of interest. In terms of being able to use the rabbit to examine the neurobiology of schizophrenia in

more detail, additional behaviors would be beneficial, both to add support to the results from EBC and to compare the rabbit to other established behavioral tests that are done in rodents. The novel object recognition (NOR) test is a popular test for declarative memory in rodents, especially for tests of schizophrenic-like impairments (44, 45, 98, 99). The test is done in two phases, an initial exploration phase where two identical objects are explored by the test animal, and a test phase that examines exploratory behavior after one of the objects has been replaced with a novel object after some period of time, e.g., 5–30 min. Rodents tend to favor the exploration of a novel object over the exploration of a familiar object, and the ratio of the time spent exploring one object relative to the other provides a cognitive index that can be evaluated.

The NOR paradigm has been used in rabbits by Hoffmann (100, 101) and was found to share similar properties with the rodent paradigm, i.e., the rabbits exhibited a preference for a novel object after a five minute delay (but not after a 20-min delay). Hoffman and colleagues also showed that acute administration of NMDA antagonists (ketamine and MK-801) significantly impaired NOR in the rabbits when the drug was administered 20 min before the sample phase of the test. The NOR paradigm in rabbits provides the opportunity to test the effects of the Meltzer paradigm for inducing schizophrenia by the chronic administration and subsequent washout of sub-anesthetic doses of NMDA receptor antagonists. Those results can then be compared directly with results from EBC studies to determine if the effects are generalized to multiple tests of memory and cognition, and if repeated doses of NMDA antagonists have prolonged effects. As noted above, the relative ease with which BOLD imaging studies can be done in rabbits offers the parallel opportunity to visualize the brain regions mediating the potential schizophrenia-like effect.

Conclusion

Trace EBC is uniquely suited to examine cerebro-cerebellar interactions since the paradigm has been shown to require both the cerebellum and the forebrain. The additional requirement for awareness of the stimulus contingencies when a stimulus-free trace interval separates the two stimuli during a trial gives the paradigm good face validity. Although the paradigm has been used most often to study neural mechanisms mediating learning and memory in healthy adults, the paradigm can be used to detect impairments in neuropsychiatric diseases, especially schizophrenia. The paradigm is also quite translational in nature and animal models of schizophrenia can be examined with EBC in several species to allow an analysis from genes to molecules to behavior. The paradigm is frequently used in rabbits, rats, mice, and humans, but the rabbit model is particularly appealing given its tolerance for restraint and the ease of using it without the need for anesthetics or sedatives during functional imaging experiments. An animal model of schizophrenia is particularly suited to answer two important questions: (1) what therapeutics are best for treating both the cognitive and psychotic aspects of schizophrenia and (2) can neuroimaging reveal biomarkers of the disease and a determination of appropriate therapeutics? Forebrain-dependent trace EBC in the rabbit is positioned to answer these questions, and the relatively new demonstration of NOR in the rabbit (100) provides an additional test for cognitive impairments and amelioration of psychotic symptoms by antipsychotic drugs.

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REVIEW
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Hippocampal Non-Theta-Contingent Eyeblink Classical Conditioning: A Model System for Neurobiological Dysfunction

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Typical information processing is thought to depend on the integrity of neurobiological oscillations that may underlie coordination and timing of cells and assemblies within and between structures. The 3–7 Hz bandwidth of hippocampal theta rhythm is associated with cognitive processes essential to learning and depends on the integrity of cholinergic, GABAergic, and glutamatergic forebrain systems. Since several significant psychiatric disorders appear to result from dysfunction of medial temporal lobe (MTL) neurochemical systems, preclinical studies on animal models may be an important step in defining and treating such syndromes. Many studies have shown that the amount of hippocampal theta in the rabbit strongly predicts the acquisition rate of classical eyeblink conditioning and that impairment of this system substantially slows the rate of learning and attainment of asymptotic performance. Our lab has developed a brain–computer interface that makes eyeblink training trials contingent upon the explicit presence or absence of hippocampal theta. The behavioral benefit of theta-contingent training has been demonstrated in both delay and trace forms of the paradigm with a two- to fourfold increase in learning speed over non-theta states. The non-theta behavioral impairment is accompanied by disruption of the amplitude and synchrony of hippocampal local field potentials, multiple-unit excitation, and single-unit response patterns dependent on theta state. Our findings indicate a significant electrophysiological and behavioral impact of the pretrial state of the hippocampus that suggests an important role for this MTL system in associative learning and a significant deleterious impact in the absence of theta. Here, we focus on the impairments in the non-theta state, integrate them into current models of psychiatric disorders, and suggest how improvement in our understanding of neurobiological oscillations is critical for theories and treatment of psychiatric pathology.

Keywords: hippocampus, neurobiological oscillations, theta rhythm, brain–computer interface, cognitive dysfunction, psychiatric disorders

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INTRODUCTION

Recent findings suggest that an estimated 18.1–36.1% of the global population will suffer from a mental disorder, as classified by the Diagnostic and Statistical Manual of Mental Disorders, during their lifetime (1). Onset of these conditions can begin as early as childhood or not appear until late adulthood. One of the primary areas affected by mental illness is cognitive functioning, including

attention and memory. Cognitive disruption is seen in a wide range of psychiatric disorders, including, but not limited to, major depressive disorder (MDD) (2), schizophrenia (3), and Alzheimer's disease (AD) (4). Due to its efficacy in both humans and animal models, eyeblink conditioning (EBC) has proven valuable as a behavioral marker of cognitive impairment in mental illness. Through studies of human patients and animal models, researchers have identified disruptions in electrophysiological activity in each of these disorders (5–8).

This review summarizes a series of findings on the relationship between theta oscillations in the hippocampus and EBC in the rabbit. We propose that EBC, which is remarkably similar behaviorally and neurobiologically in humans, can be a productive model system that can serve as a marker for psychiatric disorders and allow invasive local field potential (LFP) and single-unit analyses to investigate their neural substrates. We have developed a brain–computer interface that allows us to give training trials in the explicit presence (T+) or absence (T−) of theta in the CA1 region of dorsal hippocampus. A major feature of this interface is that, unlike drug, lesion, or genetic manipulations, our method allows the phasic increases and decreases of theta that characterize intact hippocampal function and may be a critical aspect of theta's influence on cognitive processes. We will show that EBC training in the explicit absence of theta reproduces several important behavioral and electrophysiological dysfunctions similar to what is observed in major psychiatric disorders. We argue that the electrophysiological markers at the cellular level during disordered behavioral performance will aid in our understanding of these pathologies and set the course for manipulations or treatments that can restore function or prevent the progression of disease. A major theme will be that neurobiological oscillations, especially theta, serve as important coordinators and facilitators of distributed cognitive brain systems and that the disintegration of these areas is responsible for cognitive impairment and, in extreme cases, psychiatric disorders. We conclude with recommendations for the directions such research may take.

EYEBLINK CLASSICAL CONDITIONING

Basic Behavioral Paradigm

Rabbit classical EBC is a widely used model of associative learning. It has been used in research involving humans (9) and nonhuman animals to investigate the neural substrates of associative learning (10). The EBC paradigm typically involves the presentation of a relatively neutral conditioned stimulus (CS), such as a tone, paired with, but preceding, the presentation of behaviorally relevant unconditioned stimulus (US), such as a corneal airpuff. After sufficient pairings, the subject learns to perform an adaptive eyeblink conditioned response (CR) to the CS, prior to the arrival of the airpuff US. EBC is most commonly presented in one of two general paradigms, delay or trace conditioning.

In delay EBC, the CS and US overlap and coterminate. The essential neural circuitry for delay EBC is well established and is contained within the cerebellum [for review: (11)]. The primary site of plasticity has been localized in the interpositus nucleus (IPN). Lesions of the IPN completely prevent acquisition of CRs and eliminate responding in previously trained animals without preventing eyeblinks to the UR (12). In addition to the IPN, the cerebellar cortex has also been shown to be necessary for delay EBC (13), playing a role in the precise timing and amplitude of the CR. Information about the US projects from the inferior olive (IO) to Purkinje cells in the cerebellar cortex and granule cells of the IPN via climbing fibers. CS-related information projects from the lateral pontine nuclei (LPN) to the cerebellar cortex and IPN through the mossy fiber pathway. This cerebellar pathway is essential for delay EBC acquisition and performance, but there are also structures that seem to play a modulatory role. The hippocampus, a structure strongly implicated in learning and memory, is unnecessary for learning the delay paradigm (14), though electrophysiological studies have shown conditioning-dependent changes in cellular response profiles over training (15, 16). Additionally, lesions of the amygdala have been shown to disrupt reflex facilitation in rabbits (17). Lee and Kim (18) provide evidence that the amygdala and hippocampus modulate the emotional and muscular components of EBC, respectively, interacting to allow for the overall learned behavior.

The trace form of EBC alters the paradigm by introducing a stimulus-free period between CS offset and US onset. This form of EBC still requires the cerebellar pathway discussed above (19), but lesion and inactivation studies have shown that it is influenced by the amygdala (20, 21), and requires the medial prefrontal cortex (22) and hippocampus (23). Pharmacological inactivation of the hippocampus with scopolamine, a muscarinic acetylcholine (ACh) receptor antagonist, prevented learning; however, a day of training with saline infusions resulted in a gradual acquisition of the paradigm as if training had just begun (24). Disruption of hippocampal functioning via lesions or pharmacological inactivation of major inputs has also been shown to cause behavioral deficits (25–27). Additionally, electrophysiological studies have identified conditioning-related changes in hippocampal cellular responding during the trace paradigm. Multiple-unit recordings have demonstrated gradual increases in response magnitude during the late half of the trace period as training progresses (28). McEchron and Disterhoft (29, 30) have identified several unique response profiles for hippocampal pyramidal cells at the single-unit level. The response profiles most associated with CR learning show increases in pyramidal cell firing to both the CS and US early in training; however, as the animal approaches behavioral asymptote, the response to the US, but not to the CS, begins to decrease. Additionally, recent work has shown that conditioning-related increases in single-unit firing continue through retrieval of the consolidated memory (31).

Eyeblink conditioning does not serve solely as an animal model, having been used in human subjects for over a century (32). As in rabbits, patients with cerebellar damage are impaired in learning the delay and trace forms of EBC (33–35). Those suffering hippocampal damage fail to acquire trace, but are able to learn delay EBC (9, 36–38). Additionally, neuroimaging work has implicated a role for the prefrontal cortex in trace EBC (39, 40). Due to the well-defined circuitry necessary for successful EBC performance, this paradigm is able to provide critical input into the neural regions affected in several psychiatric disorders.

Disruption of EBC in Psychiatric Disorders

Early research in patients with MDD implicated cerebellar dysfunction primarily through neuroimaging studies (41–43). The behavioral effects identified with EBC serve to corroborate regional dysfunction observed in neuroimaging studies. Training patients on both delay and trace EBC, Greer et al. (44) provided behavioral evidence indicating abnormalities in cerebellar processing. They found a significant decrease in the number of CRs in MDD patients compared to controls across both forms. While these results do not allow for differentiation of cerebellar and hippocampal dysfunction, comparison of the delay and trace paradigms has been used in other disorders to differentiate functional regions. Grillon et al. (45) compared performance on both the delay and trace EBC paradigms in patients suffering from panic disorder. There was no difference in performance between patients and control subjects on the delay task; however, patients performed significantly worse on the trace paradigm, showing a delayed acquisition rate. This pattern of results indicates hippocampal dysfunction and potential deficits in declarative memory in panic disorder patients. As panic disorder requires unexpected panic attacks, the authors posit that these deficits may underlie a patient's inability to identify predictive cues. Results have been less clear in studies of schizophrenia. Early work indicated a possible enhancement of delay EBC, with patients demonstrating faster acquisition rates than controls (46, 47). More recently, several studies have found impaired delay EBC performance through decreased acquisition rates (48–53), decreased CR amplitude (54), and less adaptively timed CRs (50) compared to controls, as well as linking those deficits to decreased cerebellar volume (49) and blood flow (52). Additionally, Marenco et al. (55) demonstrated an increase in short latency (non-adaptively timed) CRs during trace EBC in schizophrenic patients.

Eyeblink conditioning has been especially prominent in the study of AD, being used both in animal models and in human patients. Studies have shown deficits in acquisition rate for both the delay (33, 56–58) and trace paradigms (59–61), with a larger effect in the delay paradigm (59). Papka and Woodruff-Pak (62) identified the number of trials necessary to accurately assess delay EBC in AD patients, providing a more efficient test of cognitive performance that may serve as a diagnostic tool in differentiating normal aging from dementia (63). While delay EBC can be acquired normally after hippocampal removal, pharmacological disruption of the septo-hippocampal cholinergic system leads to deficits in performance (26, 64). As cholinergic disruption is a key component of AD pathology (65–67), parallel findings between rabbits with cholinergic dysfunction and AD patients provide validation of the animal model. Furthermore, galantamine, a cholinesterase inhibitor, facilitates EBC performance in aged, but not young, animals, suggesting that it counteracts the decrease in cholinergic activity associated with aging (68).

CHOLINERGIC DYSFUNCTION IN PSYCHIATRIC DISORDERS

Cholinergic systems have long been associated with cognitive functions, such as attention and memory, that are often affected in psychiatric disorders (69). The basal forebrain cholinergic system is deserving of particular attention due to the target structures of its separate cholinergic neuron populations. The first originates in the horizontal limb of the diagonal band of Broca (DBB) and nucleus basalis and projects to areas of the cortex, such as the mPFC (70), an area involved in sustained attention (71). A separate population of cholinergic projections originates in the medial septum and vertical DBB targeting the dorsal hippocampus, an essential region for encoding of declarative memory. Numerous lines of research have converged to show deficits in cholinergic functions underlying the cognitive deficits of several psychiatric disorders. In AD patients, postmortem studies have indicated a loss of cholinergic neurons in the nucleus basalis (72), a finding supported recently using MRI (73). Additionally, the primary treatments for AD involve acetylcholinesterase inhibitors as a means of increasing cholinergic activity (74–76). Other disorders linked to cholinergic dysfunction include schizophrenia and MDD. In humans, muscarinic antagonists have been shown to increase the severity and duration of both positive and cognitive symptoms in schizophrenic patients (77, 78). Furthermore, anti-muscarinics can lead to a temporary psychosis resembling schizophrenia in healthy subjects (79). Postmortem studies have shown a decrease in muscarinic ACh receptors in schizophrenia patients (80, 81). Additionally, acetylcholinesterase inhibitors have been useful in treating hallucinations (82). These findings have been corroborated in animal models where muscarinic antagonists have led to cognitive impairments and psychosis indicating behaviors in rodent models (78). Though less research has been conducted in MDD patients, recent studies have shown antidepressant effects of scopolamine, a muscarinic receptor antagonist (83), and decreased levels of muscarinic receptors in MDD. As hippocampal theta power is positively correlated with ACh activity (84, 85), it may be possible to use our model system, in which the non-theta group likely shows diminished cholinergic activity immediately preceding conditioning trials, to explore electrophysiological and behavioral bases of these disorders.

ELECTROPHYSIOLOGIOLOGICAL DISRUPTION IN PSYCHIATRIC DISORDERS

Neurobiological oscillations have been associated with memory processes, feature binding, and consciousness through their ability to synchronize across and within brain regions, though a definitive function has not been established (86–89). Synchronization of cellular activity within a region can be clearly seen in the strong relationship of single-units and neurobiological oscillations with many cells having preferred phases of the oscillation to increase their firing rates (90–93). Oscillatory potentials can be divided into a several frequency bands based on functional behaviors with which they are associated, as well as cellular and pharmacological mechanisms underlying their generation (88). It is important to note that these different oscillations do not operate in isolation, with multiple theories proposing an interaction between two frequency bands being essential for cognitive processes (89, 94, 95). As normal functioning requires the complex interplay

of oscillatory activity across brain regions, lack of synchrony or perturbations of these endogenous signals can lead to detrimental effects associated with several psychiatric disorders.

In recent years, research into causes and potential treatments for schizophrenia has increasingly emphasized a basic understanding the neural circuits and processes leading to the myriad of symptoms. Due to the large-scale network believed to be involved in the disorder, abnormalities in oscillatory dynamics seem poised to play a major role in explaining the cognitive deficits (5). At a relatively broad level, schizophrenia has been associated with alterations in the relative power of several oscillatory frequencies associated with cognitive processes, including theta (4–7 Hz), alpha (8–12 Hz), beta (15–30 Hz), and gamma (40–100 Hz) (5–8, 96, 97). Some research has also indicated the importance of understanding different frequency oscillations in the context of their cross-frequency modulation, particularly in regard to gamma and theta (97). Researchers have also attempted to examine disruptions in neural dynamics and relate them to specific disruptions of behavioral tasks (6). A common finding in electrophysiological research is phase locking of oscillatory activity following stimulus presentation, a phenomenon typically allowing for coordination of neuronal firing across a distributed system. However, schizophrenic patients have shown delays in phase locking following auditory (98) and visual stimulation (99), with the degree of phase locking correlated with the extent of visual hallucinations and thought disorders (100). Additionally, while increases in frontal midline theta are typically seen following initiation of working memory tasks (101), schizophrenic patients show no increase, and at times a decrease, of evoked theta at various degrees of working memory load (102). These disturbances have been linked to a lack of theta coherence between left frontal and temporal EEG recordings in schizophrenics compared to controls (103). At the cellular level, a loss of synchrony may affect the optimal balance between excitation and inhibition, particularly in regard to activity of GABAergic interneurons (96).

Similarly, MDD has been characterized by alterations in oscillatory activity across theta, alpha, and beta bandwidths, but has also shown decreases in delta (0.5–3 Hz) activity (104, 105). These patterns result in changes of the relative ratio of each frequency, creating a highly heterogeneous state (104). MDD patients show a convoluted pattern of effects in terms of oscillatory synchronization. While MDD is characterized by increased synchronization of alpha and beta, as well as frontal theta (105, 106), several studies have also demonstrated a decrease in frontal theta power relative to controls (107–109). Furthermore, increases in theta power following deep brain stimulation have been shown to predict long-term clinical efficacy of treatment (110). Extending beyond frontal theta, animal models of MDD have revealed the effects of theta in the medial temporal lobe (MTL). Zheng and Zhang (111) found a decrease in theta phase coupling between the ventral hippocampus and medial prefrontal cortex that was associated with decrease in synaptic plasticity of the pathway. Furthermore, Sauer et al. (112) have shown reduced synchrony of theta and gamma oscillations in the prelimbic cortex attributed in part to a decrease of output from prelimbic GABAergic interneurons.

Finally, it is important to consider neurobiological oscillations in AD, a disorder most commonly noted for the presence of amyloid beta (Aβ) plaques. Recent work has shown the potential of oscillatory activity as a means of early AD diagnosis. Compared to controls, AD patients have shown lower theta phase locking to stimuli (8), as well as decreased functional connectivity as measured by phase synchronization (113–115). Utilizing Granger causality and stochastic event synchrony, Dauwels et al. (116) demonstrated that loss of EEG synchrony can accurately predict occurrence of AD based on pre-dementia data. Using EEG synchrony as a screening tool can potentially be improved upon by applying principal component analysis before estimating synchrony (117). Animal models of AD are also being used to characterize the cellular basis of maladaptive alterations in oscillatory and cellular activity. Increasing disruption of hippocampal theta oscillations has been shown in Aβ overproducing transgenic mice as a function of age (118). Guitérrez-Lerma et al. (119) found that the two different types of hippocampal theta are affected differentially by a variety of Aβ peptides. Hippocampal pyramidal cells are disrupted in normal aging, showing a decrease in excitability over time (120, 121), as well as in AD models in which desynchronization of action potential generation leads to a shift in the excitatory/inhibitory equilibrium (122). Hippocampal Aβ also impacts functioning in target structures. For example, investigating a decrease in hippocampal theta power, Villette et al. (123) showed a reduction of firing activity in GABAergic neurons in the medial septum. Importantly, this reduction in firing was not caused by a loss of neurons, but rather an alteration in their normal firing pattern. Our model system permits analysis of specific electrophysiological responses to the conditioning stimuli in terms of LFP synchrony and cellular reactivity with precise control of hippocampal theta state.

THETA-TRIGGERED MODEL

Hippocampal Theta Oscillations

Though psychiatric disorders are accompanied by disruptions in several frequency bands, work in our lab has focused on the hippocampal theta rhythm (3–12 Hz). Across a range of species and tasks, hippocampal theta has been implicated in spatial (90, 91, 124–126), declarative (127–129), and working (101, 130, 131) memory processes. Within the theta band, Kramis et al. (132) identified two types of theta that are pharmacologically and behaviorally different, cholinergic (3–7 Hz) and non-cholinergic (8–12 Hz) theta. Cholinergic theta is present during alert immobility and is eliminated by the muscarinic ACh receptor antagonist, atropine. Non-cholinergic theta appears during voluntary movements and is unaffected by atropine. Both types of theta have been shown in the rabbit depending on the task (132, 133), with cholinergic theta being the dominant frequency during EBC.

In 1978, Berry and Thompson (134) identified a cognitive benefit of hippocampal theta that would serve as the foundation of the future development of our brain–computer interface (BCI). They found a strong positive correlation between pre-training hippocampal theta and learning rate, a finding that was recently replicated in rabbits (135) and extended into human spatial learning (136, 137). Several studies have shown that lesions to the MS reduce hippocampal theta power and significantly slow learning of an EBC task (25, 26, 64, 138). Additionally, eliciting theta

through MS stimulation or water deprivation has led to increases in learning rate (139, 140). It is important to note, however, that all of these studies utilized non-physiological alterations to the LFP, disrupting the natural ebb and flow that some believe to underlie the role of theta in cognitive processes (88, 141, 142). Also, it has been shown that artificial stimulation of the MS distorts the normal physiological response patterns of theta-related cells in the hippocampus (143). Thus, allowing the normal fluctuations of theta and non-theta states, as our interface does, may be a key to understanding the natural role of oscillations in behavioral learning and cellular response profiles.

Signal Processing Foundation of the BCI

To address that important issue, Seager et al. (144) developed a BCI capable of making training trials contingent on fluctuations in the naturally occurring oscillations. For a comprehensive overview of the BCI design and methodology, see Hoffmann et al. (145). Briefly, the BCI uses real-time spectral analysis to restrict EBCC trials to the explicit presence (T+) or absence (T−) of hippocampal theta (**Figure 1**). To accomplish this, either chronic monopolar electrodes or independently moveable tetrodes are implanted in area CA1 of the hippocampus. During training, a custom LabView program calculates a ratio of power at bandwidths specified by the experimenter. For our work that involves calculating the ratio of theta (3.5–8.5 Hz) to non-theta (0.5–3.5 Hz and 8.5–22 Hz) in real time. The ratio is calculated for 640-ms running time intervals, offset by 160 ms to allow for partially overlapping samples. In the T+ condition, a trial is triggered if the ratio of theta to non-theta exceeds 1.0 for three consecutive intervals. A trial is triggered in the T− condition if the ratio falls below 0.3 for three consecutive intervals. This methodology allows for the different training groups to receive trials under opposite theta conditions while still allowing for the natural fluctuation between trials.

Behavioral Effects of Theta-Contingent Training

The initial BCI study examined the effects of theta-contingent training during a delay EBC paradigm (144). Subjects were divided into four groups: (1) trials triggered in the explicit presence of theta (T+); (2) trials in the explicit absence of theta (T−); (3) T+ yoked controls, inter-trial intervals matched to the T+ subjects regardless of theta state; and (4) T− yoked controls. Animals trained under T− conditions learned significantly slower than those in the T+ condition (**Figure 2A**), requiring more trials to reach asymptotic performance (eight CRs out of nine consecutive trials; 8/9 CRs) and showing a lower percentage of CRs across training. Additionally, T− subjects required significantly more trials to the 8/9 criterion than their yoked controls (**Figure 2B**), highlighting the detrimental effects of T− training. This is important to note when considering non-theta-contingent training as a natural model of a dysfunctional hippocampus, as these results coincide with the previous findings that pharmacologically disrupting hippocampal functioning is more detrimental to delay EBC than having no hippocampus (64). These findings have been extended to trace EBC in several studies. Utilizing the same four groups (T+, T−, T+ yoked, and T− yoked), Griffin et al. (28) showed that T− animals required significantly more trials to reach early (fifth CR) and late (8/9 CRs) learning criteria, demonstrated a lower percentage of CRs on the first 4 days of training, and required more trials to reach fifth CR than their yoked control counterparts. These results have been replicated by our lab with T− animals reaching the fifth CR criterion later than T+ animals (146, 147) and T− animals showing a lower percentage of CRs across the first 4 days of training (148). Taken together, the deficits seen in both delay and trace EBC mirror the patterns seen in patients and animal models of several psychiatric disorders. This is particularly relevant for disorders in which the cholinergic system is affected, such as AD, as the T− condition reflects a period where the cholinergic system is not engaged.

Furthermore, our BCI findings point to a potential treatment for cognitive deficits seen in aging and AD. Asaka et al. (149) examined the effects of theta-contingent training on aged animals, those that typically show learning deficits (150, 151). Four groups of animals were trained, young T+, young yoked controls, aged T+, and aged yoked controls. As expected, aged yoked controls performed significantly worse than young yoked

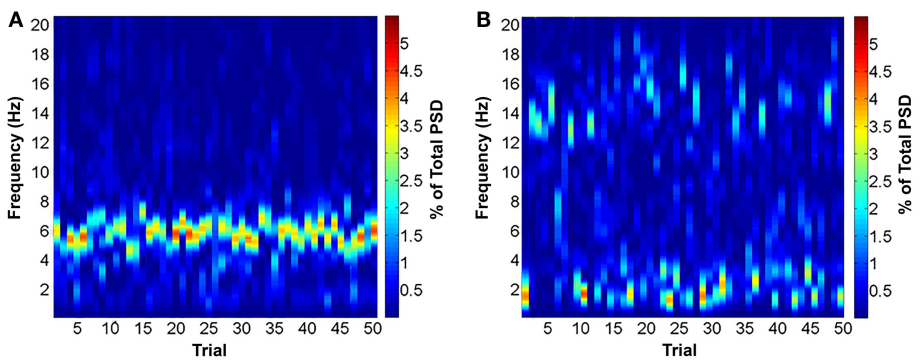

FIGURE 1 | Surface plots of power spectral density (PSD) of the pre-CS period triggering a trial for 1 day of training for an animal in the T+ (A) and T− condition (B). Trials in the T+ condition were consistently triggered under conditions of high theta and low delta and alpha. The T− condition was triggered by periods of low theta and high delta or alpha. Note that the T− condition is more heterogeneous than T+, with trials being triggered under both high delta and high alpha conditions. Figures created from data published in Cicchese et al. (146).

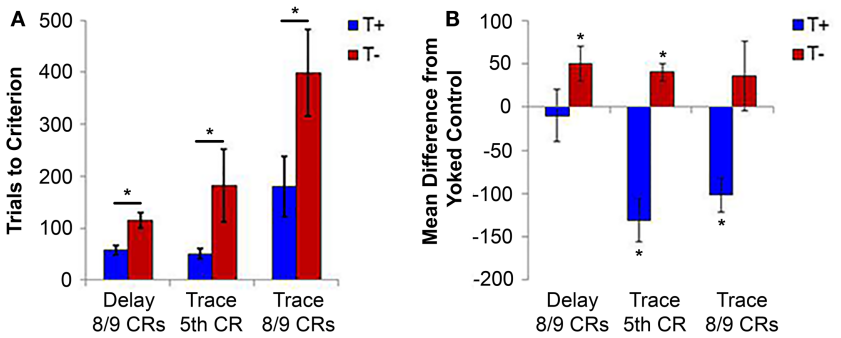

FIGURE 2 | **(A)** Average number of trials required to reach behavioral criteria in delay (8/9 CRs) and trace (fifth CR and 8/9 CRs) forms of EBC. Animals trained under T− conditions required significantly more trials than T+ animals to reach asymptotic performance (8/9 CRs) in delay conditioning, as well as more trials to reach early (fifth CR) and asymptotic (8/9 CRs) behavioral markers. **(B)** Average difference in the number of trials to reach behavioral criteria from yoked controls. T− animals needed more trials than controls to reach asymptotic performance of delay conditioning and more trials to reach the early learning criteria of trace conditioning. The differences from yoked controls provide evidence of detrimental performance in the T− condition, showing that T−/T+ differences are not simply an effect of improved performance in the T+ condition. *$p < 0.05$. Delay figures adjusted from Seager et al. (144), trace figures adjusted from Griffin et al. (28).

controls, taking longer to reach several late learning behavioral criteria (including 8/9 CRs and 80% CRs in a session). However, aged T+ animals learned significantly faster than aged yoked controls, and showed no difference in learning rate from young yoked controls (**Figure 3**). Importantly, the benefit of T+ training persisted past behavioral indicators of asymptotic performance in aged animals, suggesting that sustained accurate performance, a cerebellar-dependent function, is also affected by oscillatory state. While aging is accompanied by a decrease in cholinergic activity, the presence of 3–7 theta in the hippocampus demonstrates that periods of relatively normal cholinergic activity persist that can be engaged as a non-pharmacological intervention for cognitive deficits.

These behavioral results are consistent with recent studies in human subjects. Using magnetoencephalographic (MEG) recordings, Guderian et al. (152) found a positive correlation between pretrial theta amplitude in the MTL and recall rate in an episodic learning task. Following this demonstration, Fell et al. (153) recorded bilaterally along the longitudinal axis of the MTL with intracranial EEG. Enhancement of hippocampal theta predicted successful encoding of a word recognition task. Similarly, Lega et al. (154) recording from the hippocampus of neurosurgical patients showed higher theta power during encoding. Interestingly, the researchers identified a slow and fast center in the theta rhythm, and only the slow theta (~3 Hz) showed this pattern.

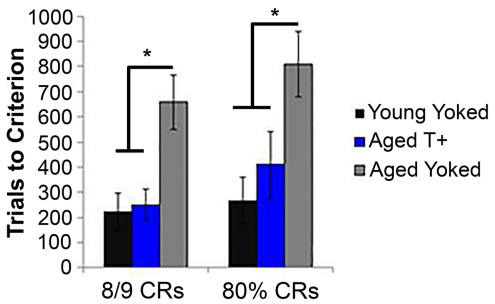

FIGURE 3 | **Average number of trials to reach the late learning criteria (8/9 CRs and 80% CRs) for young yoked controls, aged T+ triggered, and aged yoked control animals**. Aged yoked controls required more trials to reach both criteria than young yoked controls, indicating disrupted performance in aged animals. Aged animals trained under T+ conditions performed better than their yoked control counterparts and showed no difference from the young yoked controls. Thus, theta-contingent training alleviated the cognitive deficits seen in aged controls. *$p < 0.05$. Figure adapted from data published in Asaka et al. (149).

Electrophysiological Effects within the Hippocampus

In addition to deleterious behavioral effects, training in the explicit absence of theta has been shown to have negative effects on hippocampal electrophysiology at the LFP, multiple-unit, and single-unit levels. Previous work in rats has demonstrated a phase reset of the local theta rhythm following stimulus presentation (155, 156). Using the trace EBC paradigm, our lab has replicated this phase reset and shown coherent rhythmicity at theta frequencies in T+ animals following both CS and US presentation (147, 148); however, animals trained under T− conditions display a delayed onset of phase reset, as well as decreased rhythmicity in theta frequency compared to T+ animals. These results in the T− condition are important to consider as McCartney et al. (156) have shown that the phase reset produced by relevant stimuli provides ideal conditions for LTP to occur, suggesting a decrease in neural plasticity when trained in the absence of theta. Additionally, this delayed phase reset is comparable to that seen in schizophrenic patients in response to both auditory (98) and visual (99) stimuli.

Coinciding with the effects on LFPs, T− training impairs both the magnitude and rhythmicity of hippocampal multiple-units. During trace EBC, multiple-units in T− animals inhibited below baseline firing during presentation of the tone and through the 500-ms trace interval, while those in T+ animals showed

excitation (28). Note that this indicates an active suppression or inhibition of unit firing under T− conditions rather than simply the absence of an excitatory response. While this effect was seen on the second and third days of training, Darling et al. (147) linked this decrease in activity of T− units to behavioral criterion, showing significant inhibition at the early (fifth CR) and late (8/9 CRs) learning markers. Furthermore, similar to what has been seen in LFPs, T− multiple-units lack rhythmicity in firing during the trace interval, whereas T+ units fired coherently at 6.25 Hz (147).

Early work in rabbit EBC showed that conditioning-dependent changes in multiple-unit activity were the result of changes in pyramidal cell activity (16, 157). To replicate this, our theta-triggered work was continued with single-unit recordings of hippocampal pyramidal cells. To determine whether changes in multiple-unit activity were caused by large firing rate changes in a few critical cells or by a change in the overall number of cells responding in a particular way (firing rate increasing or decreasing), Cicchese et al. (146) analyzed pyramidal cell responses by their qualitative (rate increasing or decreasing) and quantitative (response magnitude) properties. Early in learning, putative pyramidal cells were more likely to decrease their firing rate during the tone period in T− than in T+ animals and more likely to increase their firing rate during both the tone and trace periods in T+ compared to T− (**Figure 4**). Importantly, there were no theta-contingent differences in the magnitude of either firing rate increases or decreases. These findings suggest that the role of theta in cellular firing is related to the recruitment of additional units firing a particular pattern, rather than a drastic change in rate of relatively few cells. This implies that an optimal hippocampal ensemble response for EBC consists of more widespread excitation of pyramidal cells rather than a sparse code of heightened responses by a few cells. Thus, theta may serve to optimize the ratio of cells showing excitation or inhibition, leading to a dysfunctional balance in the absence of theta. This conclusion would agree with findings from models of schizophrenia (96) and AD (122), implicating a shift in the excitatory/inhibitory equilibrium as a potential cellular mechanism. Additionally, Rutishauser et al. (158) found a positive correlation between performance of a memory task and coordination of hippocampal spike timing to the local theta rhythm. This is consistent with our results showing a learning deficit in T− subjects accompanied with less coherence of pyramidal cell response direction.

Electrophysiological Effects Across Brain Regions

Due to the distributed memory system involved in trace EBC, it is important to consider how non-theta-contingent training may negatively affect processing in other necessary regions. LFP recordings taken from hippocampal CA1, and cerebellar IPN and HVI, have revealed striking theta-contingent differences in both rhythmicity and synchronization between areas that may underlie dysfunctional processing during training (148). Coinciding with improved behavioral performance, T+ animals showed theta rhythmicity time-locked to conditioning stimuli in the cerebellum and precise theta antiphase (180°) synchronization between CA1 and IPN/HVI LFPs. By contrast, T− performance deficits

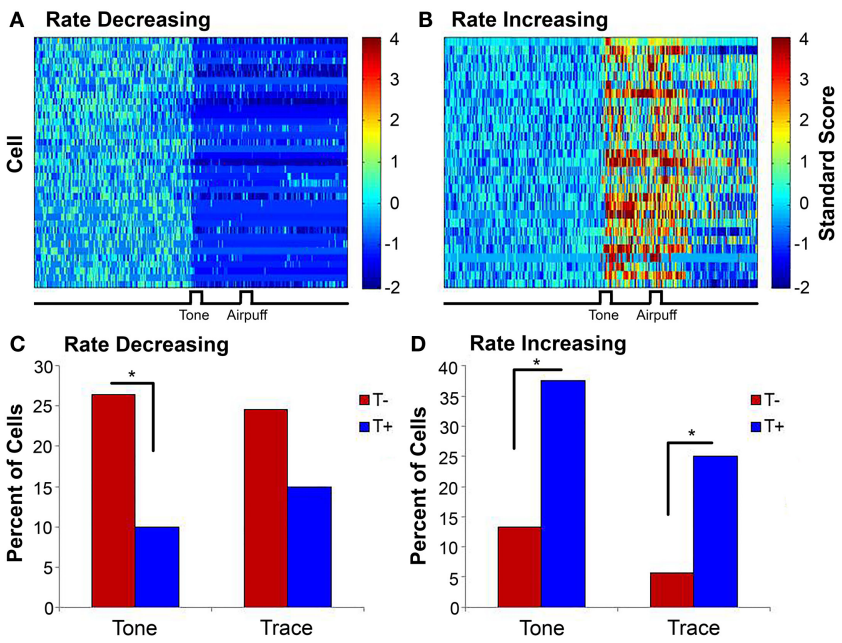

FIGURE 4 | Surface plots showing the standard scores (10-ms bins) of all rate decreasing (A) and rate increasing (B) cells averaged across the entire training session (truncated to 4 for illustration purposes). Note that rate decreases and increases during tone presentation and are sustained past airpuff presentation. **(C)** A greater percentage of cells in the T− condition were rate decreasing during the tone than in the T+ condition. **(D)** Cells in the T+ condition were more likely to increase their firing during the tone and trace periods than those in T−. *$p < 0.025$. Figure adapted from data published in Cicchese et al. (146).

were accompanied by an absence of theta oscillations in IPN and HVI, as well as a lack of synchronization with CA1. These results are consistent with human studies showing an increase in theta synchrony across distributed regions following induction of MTL theta oscillations (159), as well as with fear conditioning studies in rats showing a synchronized theta activity between the lateral amygdala and hippocampus following training (160). The lack of synchronization across areas is of particular interest in light of psychiatric research. Animal models of MDD have implicated the absence of ventral hippocampus–mPFC theta phase coupling with decreased synaptic plasticity (111), while a loss of cortical EEG synchrony is a fundamental feature in AD (113–116). These oscillatory disruptions likely cause a decline in functional connectivity, failing to coordinate activity across regions necessary for cognitive processes.

The hippocampus does not directly project to the cerebellum, but may have an indirect influence through its effects on the mPFC. The mPFC is necessary for trace EBC (161, 162) and projects to the lateral pontine nucleus, which conveys important CS-related mossy fiber input to the cerebellum (163). Previous work has identified a mPFC cellular response profile characterized by inhibition followed by a period of persistent excitation in response to tone presentation (164). This pattern is thought to increase the salience of the tone by increasing the signal-to-noise ratio. Darling et al. (147) capitalized on our theta-triggered paradigm by recording simultaneously from area CA1 and the mPFC (caudal anterior cingulate region) under T+ and T− conditions. Interestingly, though the inhibitory/excitatory pattern was replicated in T+ animals, it was absent in those trained under T− conditions. This finding implies that mPFC processing is highly related to hippocampal theta state and that our T− animals may fail to apply proper motivational salience to the conditioning stimuli. Importantly, the increased theta synchrony between hippocampus and amygdala during Pavlovian conditioning (160) raises the possibility that motivational and emotional input from the basolateral amygdala normally converges on the mPFC in synchrony with hippocampal input to modulate salience; thus, in the absence of hippocampal theta, a lack of converging input disrupts processing of the stimuli. A similar effect is seen in schizophrenia where patients show maladaptive motivational salience when rating reinforcements (165) and when learning to discriminate between a predictive CS+ and neutral CS− (166, 167). Additionally, compared to controls, schizophrenia patients show increased neural activity to the CS− in regions associated with learning (166, 167). Thus, our T− condition appears to replicate some important findings from the human literature and relate them to neuronal response patterns in important structures.

CONCLUSION

Summary and Limitations

As the study of cognitive processes has moved away from discrete functional regions to distributed neural networks (168), it is essential to understand the oscillatory activity capable of synchronizing these anatomically disparate regions (88, 141, 142). Similarly, a focus on electrophysiological disruption in psychiatric disorders is proving invaluable as loss of synchronization across regions is a common feature underlying their pathology (8, 112–114). Using our BCI, we have shown that training in the explicit absence of hippocampal theta produces deficits in EBC expected of a number of psychiatric conditions. Furthermore, these behavioral deficits are accompanied by electrophysiological disruptions at the LFP (147, 148), multiple- (28, 147), and single-unit (146) levels that are characteristic of conditions as disparate as schizophrenia, MDD, and AD. Of particular interest are the patterns seen across the regions necessary for EBC, with a lack of synchrony between hippocampus and cerebellum (148) and the absence of relevant response patterns in mPFC units (147). Though our non-theta-triggering has proven effective at modeling the electrophysiological correlates of a disrupted system, it is important to note that it still has room to grow. The BCI allows for trials to be delivered in the presence of a specific brain state, but does not give control of that activity. Thus, fluctuations in pretrial activity that may typically be abnormal in disorders cannot be controlled for. However, the ability of our non-theta-triggering to model interruption of distributed neural networks without lesions or pharmacological intervention provides a tool for studying psychiatric disorders in a more natural way, allowing for decreased levels of the given frequency, as is typical in illness, rather than complete abolition.

An important challenge to our findings has recently been published in the form of a failure to replicate the benefits of theta-contingent EBC (169). The authors found that animals trained under T− conditions were more likely to acquire the paradigm than yoked controls or those trained in the presence of theta; however, it should be noted that T− animals required more sessions to reach behavioral criterion than their yoked controls, consistent with our findings. These findings seem to contradict numerous studies in animals (28, 135, 144, 146–148) and humans (152–154), showing beneficial learning effects of increased hippocampal/MTL pretrial theta. Due to a fundamental methodological difference, it is possible that the study by Nokia and Wikgren (169) does not directly apply to our work. Specifically, in their study, all subjects were presented with a full session of unpaired conditioning before training began. This introduces latent inhibition as a major confound to later learning effects. While T+ and T− animals each received the unpaired session, work has not been completed to investigate how effects of latent inhibition may interact with theta-contingent learning conducted after unpaired presentations. For example, unpaired presentations of CS and US have been shown to cause a baseline EEG shift from pre- to post-exposure (170), and latent inhibition produces significantly reduced hippocampal unit responsiveness to a tone CS (171). An effect of the unpaired session is suggested by the unusually low percentage of animals that successfully acquired the CS–US association. Additionally, T+ animals that reached criterion took an average of ~5 fewer sessions than their yoked control counterparts; however, that difference was not significant, likely due to insufficient power (T+: $n = 4$, yoked control: $n = 2$; $0.05 < p < 0.10$). While these results highlight the complex relationship between oscillatory potentials and different learning paradigms, potential differences in hippocampal functioning caused by latent inhibition, as well as low statistical power, prevent a direct comparison to our theta and non-theta-contingent findings.

Future Directions

Knowing the established effect of theta on cognitive processes, it will be critical to further study its role. In particular, further exploration of mPFC theta activity could serve to bridge the gap between animal and human recording studies. Much of the theta work in human subjects has centered on frontal midline theta, but it is still unclear what the neural correlates underlying these oscillations are (101). By understanding the relationship between oscillations in subcortical structures and those recorded by scalp EEG, it would be possible to utilize neurofeedback training as a possible treatment for psychiatric conditions, similar to what has been done in patients with ADHD (172, 173).

Though our BCI does not allow for direct manipulations of theta, new research methods, such as optogenetics, may make this possible. Using optogenetic stimulation of the medial septum could provide precise temporal control of theta rhythm induction. During this stimulation, simultaneous recordings from relevant areas (hippocampus, mPFC, and cerebellum) could provide further insight into the electrophysiological relationship of the distributed network. Specifically, this methodology would allow for precise control over theta phase during stimulation presentation. Considering the prominent model of separate encoding and retrieval phases of theta (128), our T+ group could be further studied by looking at trials triggered consistently on either the peak or through of theta. It is possible that triggering during the retrieval phase of the theta rhythm could be equally detrimental to training in the absence of theta, an idea recently supported using theta-contingent training in conjunction with threshold values to target specific phases (174). Furthermore, optogenetic manipulation of theta state could be used in conjunction with conditional genetic knockout animal models to identify potential benefits of inducing synchronous neural activity in animals that are typically lacking. Initial studies into this possibility could utilize classical conditioning to allow for discrete learning points. By doing so, optogentic stimulation of the medial septum at theta frequency could be initiated prior to CS delivery, ensuring synchronous and homogeneous neural activity when learning is expected to occur. Dependent on the results, additional work should be completed to examine the amount of time asynchronous activity must be disrupted for alleviation of behavioral deficits. While research has shown physiological difference in cellular responding to naturally occurring and artificially stimulated theta (143), it is likely that optogentically induced theta would still provide benefits in animals with genetically disrupted theta oscillations. Several studies using the Morris water maze support this notion. Deficits in performance caused by disruption of hippocampal theta via pharmacological inactivation of the medial septum (175–177) or fimbria-fornix lesions (178) were overcome by artificial stimulation at theta frequency. Conversely, recent contextual fear conditioning work found a decrease in performance as a result of artificial theta stimulation (179). The authors propose, however, that the continuous stimulation provided at a fixed frequency may have interrupted the normal oscillatory processes of the rat; specifically, the constant theta likely interfered with the natural theta entrainment experienced during walking and sniffing as the rat explores its environment. Furthermore, they suggest that stimulation coinciding with an external cue, such as a tone CS, may show enhancement in performance similar to the aforementioned studies.

Although our work has focused on the theta to non-theta [3.5–8 Hz/(0.5–3.5 Hz + 8.5–22 Hz)] ratio, the LabView program can be set with any frequency range in the numerator and denominator. With this flexibility, future studies could utilize the BCI for training contingent on different frequency bands and exploration of different definitions of non-theta. Our non-theta state is heterogeneous, with major contributions of delta (0.5–2 Hz) and alpha (8–12 Hz) compared to the homogeneous theta band. This heterogeneity may underlie the detrimental effects seen in our non-theta conditioning. It will be important for future studies to alter the frequencies defined as non-theta, including using individual frequency bands in the denominator, to determine whether the decrease in theta or the heterogeneity of oscillatory bands is responsible for adverse learning. In work by others, triggering trials based on sharp-wave ripple oscillations (150–250 Hz) has been shown to increase EBC learning rate and increase the phase locking of theta oscillations to conditioning stimuli (180), suggesting that the heterogeneity of our non-theta state plays an important role. Therefore, it will be important to continue research into the effects of ripple-contingent training and their relation to theta. As discussed previously, several frequency bands are disrupted in psychiatric disorders. In light of the differences in behavioral and neurochemical characteristics of these various oscillations, it is critical to understand the contributions of each to cognitive processes and psychiatric pathology. Multidisciplinary approaches as discussed above will be an important contributor to this effort.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Timing Tasks Synchronize Cerebellar and Frontal Ramping Activity and Theta Oscillations: Implications for Cerebellar Stimulation in Diseases of Impaired Cognition

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Timing is a fundamental and highly conserved mammalian capability, yet the underlying neural mechanisms are widely debated. Ramping activity of single neurons that gradually increase or decrease activity to encode the passage of time has been speculated to predict a behaviorally relevant temporal event. Cue-evoked low-frequency activity has also been implicated in temporal processing. Ramping activity and low-frequency oscillations occur throughout the brain and could indicate a network-based approach to timing. Temporal processing requires cognitive mechanisms of working memory, attention, and reasoning, which are dysfunctional in neuropsychiatric disease. Therefore, timing tasks could be used to probe cognition in animals with disease phenotypes. The medial frontal cortex and cerebellum are involved in cognition. Cerebellar stimulation has been shown to influence medial frontal activity and improve cognition in schizophrenia. However, the mechanism underlying the efficacy of cerebellar stimulation is unknown. Here, we discuss how timing tasks can be used to probe cerebellar interactions with the frontal cortex and the therapeutic potential of cerebellar stimulation. The goal of this theory and hypothesis manuscript is threefold. First, we will summarize evidence indicating that in addition to motor learning, timing tasks involve cognitive processes that are present within both the cerebellum and medial frontal cortex. Second, we propose methodologies to investigate the connections between these areas in patients with Parkinson's disease, autism, and schizophrenia. Lastly, we hypothesize that cerebellar transcranial stimulation may rescue medial frontal ramping activity, theta oscillations, and timing abnormalities, thereby restoring executive function in diseases of impaired cognition. This hypothesis could inspire the use of timing tasks as biomarkers for neuronal and cognitive abnormalities in neuropsychiatric disease and promote the therapeutic potential of the cerebellum in diseases of impaired cognition.

Keywords: eyeblink conditioning, interval timing, ramping activity, theta oscillations, cerebellum, prefrontal cortex

INTRODUCTION

Timing is highly conserved for all mammals, and although it is paramount to survival, the precise neural mechanisms underlying the perception of time are unknown. Depending on the duration of time and type of behavioral task, the frontal cortex, striatum, hippocampus, and the cerebellum have been implicated in timing (1, 2). Neuropsychiatric illnesses such as Parkinson's disease (PD), autism, and schizophrenia involve cognitive impairment (3, 4). Mammals depend on time for working memory, attention, reasoning, communication, decision-making, and movement. As a valid proxy for cognition, timing tasks present a window into aberrant neural circuitry in animal models and in human neuropsychiatric disease (4–6).

The seminal theories of cognitive dysfunction in neuropsychiatric disease indicate a disruption in the fluid and coordinated sequences of thought and action that are the hallmarks of normal cognition (7, 8). Based on consistent abnormalities in structural and functional imaging of schizophrenia, cognitive dysmetrias are thought to occur as a result of abnormalities in a network between the cerebellum and frontal cortex (7, 8). The network connecting the frontal cortex and cerebellum involves an efferent disynaptic projection via corticospinal tracts to the ipsilateral rostral pontine nuclei (9). The afferent cerebellar projection is through the ventrolateral and mediodorsal thalamic nuclei (10–13). Cerebellar stimulation dynamically influences the medial frontal cortex in animals (14–16) and is safe and effective in alleviating cognitive impairments and elevating mood in patients with schizophrenia (17). Therefore, the pathway between the cerebellum and medial frontal cortex could be isolated to investigate cognitive circuitry and the therapeutic potential for cerebellar stimulation in diseases involving compromised cognition.

TIMING TASKS REQUIRE COGNITIVE PROCESSING IN THE CEREBELLUM AND FRONTAL CORTEX

Eyeblink conditioning and interval timing are two tasks requiring temporal processing that can be used in animals and humans to investigate the cerebellar influence on the frontal cortex. Eyeblink conditioning is the canonical paradigm to investigate cerebellar function as timing is impaired following cerebellar inactivation and lesion (18–24). Additionally, eyeblink conditioning is a powerful technique to illuminate cerebellar dysfunction in neuropsychiatric disorders (25–28). Eyeblink conditioning involves the pairing of a neutral conditioned stimulus (CS), such as a light or tone, with an aversive unconditioned stimulus (US), typically an airpuff to the eye or periorbital shock, to elicit an unconditioned response (UR). Following repeated pairings of the CS and US, the subject adaptively predicts the pending US and elicits a preventative conditioned eyeblink response (CR) that precedes the onset of the US. Two types of eyeblink conditioning exist in which there is either no interval between the CS and US and the two stimuli co-terminate (delay conditioning) or an interval of time between the two so that the offset of the CS is several milliseconds or seconds before the onset of the US (trace conditioning). Although studies claim trace and delay conditioning recruit different brain regions, they both involve activity in the cerebellum and medial frontal cortex (29–31).

This is an important consideration because the cerebellum and medial frontal cortex are both essential for accurate timing and both are aberrant in neuropsychiatric disease (25–27, 30–34). Although eyeblink conditioning involves motor performance, timing the interval also requires working memory, attention to time, and therefore involves cognitive processing. Animals with a disrupted cerebellum (35) and humans with cerebellar damage exhibit spared motor performance while eyeblink conditioning is impaired (36), indicating a separate role of the cerebellum in cognitive and motor function. Additionally, PET imaging studies indicate that both the frontal cortex and cerebellum are involved in eyeblink conditioning (37, 38) and they are hypoactive concurrent with impairments in eyeblink conditioning in patients with schizophrenia (26, 27).

Interval timing closely resembles eyeblink conditioning in that two stimuli are separated by an interval of time, and subjects estimate the passage of the specified interval. Subjects hold temporal information regarding the passage of time in their mind while they estimate when the respective amount of time has elapsed by making a motor response. Interval timing critically depends on the medial frontal cortex, which is impaired in patients with neuropsychologic illness (25–27, 30–34). There are currently no studies in animals reporting a cerebellar involvement in interval timing, likely due to the traditional view of cerebellar contributions to only subsecond temporal processing (2). However, humans with cerebellar damage have profound deficits discriminating longer intervals (8–32 s) in a temporal bisection task (39). Therefore, the cerebellum merits further investigation during interval timing tasks that require timing in the range of seconds. By combining interval timing literature with the work on eyeblink conditioning, we could gain insight into the function of cingulocerebellar circuitry and its dysfunction in cognitive disease.

TIMING TASKS CAN BE USED TO PROBE THE NEURAL MECHANISMS UNDERLYING COGNITIVE PROCESSING

Although different timescales are often used, there are two types of neuronal activity that are consistently described during timing tasks: ramping (consistent increases or decreases in neuronal firing) (40–48) and low-frequency oscillations (42, 43, 49, 50). Single medial frontal cortical neurons that are consistently active or increase or decrease activity to bridge the interval between the CS and US are consistently reported during operant and classical conditioning paradigms, including eyeblink conditioning (9), interval timing (42), and fear conditioning (51). These neurons are often referred to as climbing, bridging, or ramping neurons, but we will refer to them as ramping neurons in this manuscript.

Ramping activity involves the accumulation of temporal information between the stimuli encoding the start of the trial, US or reward availability, and response time. Of these ramping neurons, 15–20% of them encode the passage of time by ramping or accumulating the increase or decrease in action potentials

over a behaviorally relevant timing window (9, 52). Although essential to bridge the CS and US, this activity may indicate when to respond prior to the end of the CS in delay conditioning. A subset of cerebellar neurons shows a similar pattern of bridging or ramping activity to that of frontal neurons during eyeblink conditioning (9, 53, 54). Therefore, it is speculated that consistent activity in the medial frontal cortex provides the cerebellum with timing information for bridging the temporal gap between the CS and US regardless of the presence of an interstimulus interval (9).

Ramping activity that reverberates throughout the circuit could represent timing as a circuit-wide phenomenon rather than structure and task specific. Investigating concurrent medial frontal and cerebellar activity during timing tasks in healthy and aberrant states could elucidate how the brain encodes cognitive processes. Neuronal activity that lapses the interval could represent working memory processes. Therefore, combining the literature from both the eyeblink conditioning and interval timing fields could provide a circuit-based interpretation of how the brain encodes time and incidentally, cognition.

In addition to the role of ramping activity during timing, cue-evoked theta activity is also essential for temporal processing (42). During interval timing, rodents and humans have similar bursts of low-frequency activity immediately following trial start (42, 43) as measured by multi-neuron local field potential (LFP) signals. This burst of cue-evoked activity could represent the start of an internal clock in timing tasks that initiates ramping activity in single neurons to encode the passage of time (50). Low-frequency oscillations also synchronize activity within brain networks as revealed by coherence in theta frequencies between brain areas, presenting a mechanism for how neuronal networks organize behavior across time (55).

Concomitant with ramping patterns, medial frontal theta activity is dependent on dopamine as revealed by diminution of low-frequency oscillations following focal D1 dopamine blockade in the frontal cortex during interval timing (42, 43). PD characteristically involves dopamine dysfunction, and consistent with these results, medial frontal theta activity is attenuated in PD patients (43). We previously described common mid-frontal oscillations triggered by the cue (tone) during interval timing tasks in both humans and rodents (43). Additionally, the prelimbic cortex and cerebellar nuclei are coupled at low frequencies (56, 57). Synchronization between ramping neurons in both the cerebellum and frontal cortex during cognitive processing indicates that rather than one area encoding time, low-frequency activity throughout a circuit may be essential, implicating a highly conserved neural architecture for temporal organization of behavior in mammals.

CEREBELLAR STIMULATION DURING TIMING TASKS CAN BE USED TO RESCUE NEURAL MECHANISMS UNDERLYING COGNITION

If cerebellar and frontal areas both encode cognitive processes, cerebellar stimulation could be used to recover aberrant neuronal activity and rescue cognitive abnormalities in disease. Cerebellar vermal transcranial magnetic stimulation (TMS) produced downstream changes in neuronal activity in the frontal cortex as revealed by electroencephalogram (EEG) (58). A classic study by Cooper et al. electrically stimulated the cerebellum in patients with epilepsy and reported improved cognition based on increased alertness, improvement in thinking, and fluency of speech in addition to many enriched emotional characteristics (59). Recently, cerebellar theta-burst (TMS) was reported to be safe and effective in alleviating some cognitive impairments and elevating mood in treatment-resistant schizophrenia patients (17). There are currently several clinical trials further investigating the therapeutic potential of the cerebellum in schizophrenia, yet the underlying neuronal mechanisms remain unknown – Clinicaltrials.gov (60). These studies indicate that there is great potential for cerebellar stimulation to be used to treat cognitive symptoms of neuropsychiatric disease pending the explicit mapping and understanding of the influence of the cerebellum on frontal circuits.

Cerebellar dentate electrical stimulation has been shown to influence the dopamine efflux in the frontal cortex (14–16, 61). Conversely, electrical stimulation of the prelimbic frontal cortex elicited neuronal firing in cerebellar lobule VII (61) establishing a physiologic mechanism for communication between the two areas. However, to our knowledge, cerebellar stimulation has never been explored in behaving animals. We recently described a novel method to use cerebellar optogenetic stimulation to rescue cognitive deficits induced by pharmacological frontal inactivation in behaving animals. In addition to providing critical information regarding aberrant neural circuitry in disease, cerebellar stimulation can be used to recover dysfunctional neurons and rescue timing impairments in eyeblink conditioning and interval timing tasks.

CLINICAL IMPLICATIONS

We have hypothesized that cognitive processing during timing tasks relies on low-frequency, cue-evoked activity in the medial frontal cortex to signal the start of single neuron ramping. Ramping activity could represent an internal clock encoding the passage of time and indicating when to make a motor response (41, 42). By combing frontal EEG with cerebellar TMS, we can investigate how cerebellar stimulation influences neuronal activity in the frontal cortex. We hypothesize that low-frequency cerebellar stimulation will reinstate both low-frequency oscillations and ramping properties of medial frontal neurons in patients with neuropsychiatric illness.

Electroencephalogram activity indicates the sum of a large population of neurons over a relatively poor spatially represented area. This technique will allow us to investigate neuronal oscillations in humans, but only rodent models can be used to investigate how stimulation influences ramping activity. We recently explored temporal processing in PD (43). PD involves the death of dopaminergic neurons in the substantia nigra, pars compacta and in the ventral tegmental area that projects to the frontal cortex (62). We hypothesized that dysfunctional frontal dopamine would lead to diminished frontal theta and result in impaired interval timing performance. We recorded EEG from patients with PD and healthy controls while they performed

interval timing tasks, and to explore ramping activity, we used an animal model of frontal dopamine depletion with 6-OHDA in the medial frontal cortex. Interestingly, patients with PD and animal models of PD have diminished oscillations during interval timing tasks and ramping activity is diminished concurrent with dysfunctional temporal processing (43). These data indicate specific dopamine-dependent activity in the medial frontal cortex is necessary for interval timing and therefore, cognitive processing.

We hypothesize that cognitive abnormalities are similar between many neuropsychiatric diseases including PD, schizophrenia, and autism. In schizophrenia, the prefrontal cortex shows abnormal D1 dopamine (63–65), and patients inaccurately estimate time (66, 67). Cerebellar TMS has been shown to decrease negative symptoms including cognitive processing in patients with schizophrenia (17). However, if cerebellar stimulation is to become a useful treatment strategy targeted at currently untreatable cognitive impairments in schizophrenia, the precise neuronal effects of cerebellar stimulation need to be illuminated. Reinhart et al. recently reported that patients with schizophrenia have impaired frontal theta activity and cerebellar stimulation appears to rescue this activity (55, 68). The therapeutic potential of cerebellar stimulation during timing tasks has never been studied. Thus, combined TMS and EEG neural recordings in patients with PD, schizophrenia, autism can be used to investigate the neural mechanisms underlying cognitive processing during timing tasks. Cerebellar stimulation is currently in clinical trials to be used to treat the recurrent cognitive symptoms of schizophrenia. Therefore, we expect that insights from this research will guide future therapies for devastating neuropsychiatric diseases. Performance on timing tasks and frontal dysfunction may be a useful clinical biomarker of frontal dysfunction in neuropsychiatric illness.

CONCLUSION

Eyeblink conditioning and interval timing are powerful techniques that can be used in both human and animals to probe cognitive processing in cerebellar and frontal cortical circuitry. Timing tasks can provide us with a behavioral outcome to evaluate the efficacy of cerebellar stimulation on the frontal cortex neuronal activity and cognitive processing neuropsychiatric diseases including schizophrenia, bipolar disorder, ADHD, autism, OCD, and PD. As EEG is widely available, inexpensive, and easily executed, the detection of diminished frontal theta has the potential to be used as a biomarker of neuropsychiatric cognitive and neuronal dysfunction (28). TMS is rapidly becoming an important research tool in neuropsychiatric illness (60), so identifying a specific type of activity that encodes timing and cognition could guide individualized stimulation according to abnormalities in real time. Specifically, a closed-loop design where cerebellar stimulation is based on real time, aberrant frontal activity as defined by a temporal prediction error, could inspire a new paradigm to adaptively stimulate cerebellar neurons using TMS with temporal specificity to reinstate accurate timing and cognitive processes (69).

AUTHOR CONTRIBUTIONS

KP takes full authorship of this manuscript.

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REVIEW
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Eyeblink Conditioning in Schizophrenia: A Critical Review

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There is accruing evidence of cerebellar abnormalities in schizophrenia. The theory of cognitive dysmetria considers cerebellar dysfunction a key component of schizophrenia. Delay eyeblink conditioning (EBC), a cerebellar-dependent translational probe, is a behavioral index of cerebellar integrity. The circuitry underlying EBC has been well characterized by non-human animal research, revealing the cerebellum as the essential circuitry for the associative learning instantiated by this task. However, there have been persistent inconsistencies in EBC findings in schizophrenia. This article thoroughly reviews published studies investigating EBC in schizophrenia, with an emphasis on possible effects of antipsychotic medication and stimulus and analysis parameters on reports of EBC performance in schizophrenia. Results indicate a consistent finding of impaired EBC performance in schizophrenia, as measured by decreased rates of conditioning, and that medication or study design confounds do not account for this impairment. Results are discussed within the context of theoretical and neurochemical models of schizophrenia.

Keywords: schizophrenia, cerebellum, eyeblink conditioning, associative learning, cognitive dysmetria

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INTRODUCTION

Growing empirical evidence suggests cerebellar abnormalities in schizophrenia. In terms of cerebellar morphology, imaging studies report reduced cerebellar volume in chronic (1–4), neuroleptic-naïve (5), adolescent (6), first-episode (7–9), and childhood-onset (10) schizophrenia [for exceptions see Ref. (11, 12)]. Postmortem studies have also found reduced size and density of Purkinje cells in schizophrenia (13–15). In addition to structure, cerebellar function has also been reported to be abnormal in schizophrenia. Functional neuroimaging studies report abnormal cerebellar activation at rest (16–18) and during cognitive tasks [Ref. (19–21); see Ref. (22) for critical review] in individuals with schizophrenia.

These structural and functional cerebellar abnormalities appear to have clinical and functional implications in schizophrenia. Specifically, cerebellar abnormalities are associated with clinical symptoms, cognitive deficits, and outcome measures in schizophrenia (3, 23–25). For example, deficits in working memory and mental flexibility correlate with cerebellar volume (26), and fronto-cerebellar metabolic abnormalities are associated with anhedonia and ambivalence (27). Moreover, increased connectivity between frontal–parietal and cerebellar regions predicts better cognitive performance in controls and individuals with schizophrenia, and individuals with schizophrenia with improved connectivity have fewer disorganization symptoms (28).

These empirical findings are often integrated into the cognitive dysmetria theory of schizophrenia, which places the cerebellum prominently in the cortico-cerebellar-thalamic-cortical

circuit (CCTCC). The theory of cognitive dysmetria proposes a model of schizophrenia wherein deficits in this circuit are associated with both motor dysfunction and the clinical presentation of schizophrenia, and abnormalities in the CCTCC are believed to mediate the disordered cognition, behavior, and motor function characteristic of individuals with schizophrenia (29). A behavioral measure of cerebellar integrity, such as eyeblink conditioning (EBC), that can be administered to individuals with schizophrenia as an index of how well the cerebellum and interrelated circuits are performing is vital to the investigation of the cerebellum as a critical node in the CCTCC and locus of dysfunction in this influential theory of schizophrenia.

Eyeblink conditioning is a widely used measure of cerebellar-dependent associative learning. In the delay form of this task, a conditioned stimulus (e.g., brief tone) is paired, and co-terminates, with an unconditioned stimulus (e.g., air puff to the eye) that elicits an unconditioned response (e.g., eyeblink). Over the course of repeated paired presentations, a conditioned eyeblink response (CR) occurs in response to the tone and preceding the onset of the unconditioned stimulus. EBC is used in the study of clinical disorders such as schizophrenia and autism as well as aging for several reasons. First, the neural circuit underlying EBC has been well-characterized in non-human animals, with the specific brain stem nuclei associated with both stimulus encoding and motor output remarkably well-understood [see Ref. (30), for review]. Furthermore, the neural plasticity underlying standard delay EBC has been localized to the ipsilateral dorsal lateral anterior interpositus nucleus, and specific areas of the cerebellar cortex involved with timing and gain control of the conditioned response have also been identified [again see Ref. (30), for review]. Second, the conditioned response that develops over the course of delay EBC is well-preserved across species including rodents [e.g., Ref. (31, 32)], rabbits [e.g., Ref. (33)], cats [e.g., Ref. (34)], and humans [e.g., Ref. (35)], making EBC a widely used translational probe of cerebellar function. Finally, the associative learning induced by EBC is a non-declarative form of learning that occurs outside of intention and conscious awareness (35). Because performance on EBC is not dependent on higher-order cognitive function or the ability to follow complex instructions, it can be studied in individuals across a variety of ages and clinical presentations.

Importantly, the robust identification of cerebellar circuitry underlying delay EBC in non-human species is remarkably consistent with human EBC findings. Such evidence has emerged from studies involving patients with cerebellar lesions, dual-task interference, transcranial direct current stimulation (tDCS), and functional brain imaging. Specifically, individuals with cerebellar strokes demonstrate impairments in delay EBC performance (36–38). In addition, studies have demonstrated a significant relationship between performance on delay EBC and cerebellar-dependent timed interval tapping (39) as well as dual-task interference during simultaneous delay EBC and timed interval tapping (40) in non-psychiatric controls. tDCS applied to the cerebellum during acquisition has been shown to modify delay EBC performance (41). Finally, human brain imaging studies investigating the neural substrates of EBC converge with the lesion and dual-task studies described above, as well as further localize the site of EBC learning-related plasticity in humans. Specifically, positron emission tomography (PET) studies have revealed changes in cerebellar activation during EBC (42–46), and functional magnetic resonance imaging (fMRI) BOLD activation changes in the cerebellum are consistently reported during EBC (47–50).

In the first published review of EBC studies and schizophrenia (51), the author concluded that overall the EBC findings were inconclusive and any observed EBC deficits may be accounted for by antipsychotic medication administration. Lubow (51) called for an explicit comparison between medicated and non-medicated individuals with schizophrenia. In addition, concerns were raised about drawing firm conclusions regarding EBC impairment in schizophrenia due to inconsistencies in the analysis of EBC (i.e., whether or not studies accounted for alpha responses and spontaneous blink rate), possible group differences in processing and encoding EBC stimuli, the notorious heterogeneity present in the diagnostic category of schizophrenia, and the small sample sizes and disproportionate number of male individuals with schizophrenia reported in the literature (51).

Two subsequent brief reviews have appeared as subsections in two recently published articles, one reviewing EBC performance across many neurodevelopmental disorders (52) and another reviewing cerebellar-related motor dysfunction in schizophrenia and high-risk populations (53). The authors of both brief reviews largely emphasized the emerging pattern of abnormal EBC performance in schizophrenia, citing the large sample sizes and the persistent deficit in EBC performance in an unmedicated subsample reported in studies published after Lubow's (51) review (52), as well as even more recent studies of EBC impairment in individuals with schizotypal personality disorder, first-degree relatives of individuals with schizophrenia, and individuals with schizophrenia who are medication-free for a period of several weeks (53). However, both groups also acknowledged the possible role of antipsychotic medication and methodological variability in the inconsistent findings across studies (52, 53).

Importantly, since the publication of Lubow's (51) initial review of nine articles, six additional studies have been published examining EBC in the schizophrenia spectrum. These six studies account for 48% of all individuals in the schizophrenia spectrum that have participated in delay EBC studies, nearly doubling the number of participants in the schizophrenia spectrum that have been studied since Lubow's (51) review. However, questions still persist regarding the source of inconsistency in the literature examining EBC in schizophrenia, specifically related to the potential effects of antipsychotic medication and heterogeneity in methodology.

The purpose of the present review was to conduct a thorough and integrative review of published studies of EBC in the schizophrenia spectrum. Given Lubow's (51) findings and cautions as well as the conclusions of Reeb-Sutherland and Fox (52) and Bernard and Mittal (53), special attention was paid to (1) evidence of antipsychotic medication effects, (2) inconsistencies between studies in and any systematic effects of stimulus and analysis parameters, and (3) differences in sample size and sample characteristics. Finally, the findings of this review are interpreted within the context of existing models of schizophrenia.

METHOD

Tables 1–5 catalog 15 studies examining EBC in individuals with schizophrenia. These studies were first identified using Lubow's existing review of EBC in schizophrenia. Studies examining EBC in the schizophrenia spectrum published subsequent to this review were identified using PubMed, a resource of the National Center for Biotechnology Information (NCBI), at the National Institutes of Health's (NIH) U.S. National Library of Medicine (NLM).

Various domains of information from these 15 studies examining EBC in the schizophrenia spectrum were then recorded and organized, including sample characteristics (see **Table 1**), parametric properties of the EBC tasks and analyses, and major findings (see **Tables 2–5**). In the review of this literature, careful attention was paid to (1) findings that occur consistently across studies and across research groups, (2) the relationship of medication status to consistent findings, (3) any sample characteristics or parametric variability (in either EBC paradigms or analyses) that may contribute to heterogeneity of findings, (4) correlates of EBC performance in individuals along the schizophrenia spectrum, and (5) the implications of the findings of this review for current systems-level and neurobiological theories of schizophrenia.

RESULTS

Conditioning

Conditioned Responding (e.g., %CRs)

Of the 15 studies of delay EBC in schizophrenia, 9 demonstrated decreased CRs compared to controls (58, 61–68), 4 found no group differences in rates of conditioned responding (54, 55, 59, 60), and 2 reported facilitated conditioning in schizophrenia (56, 57). It should be noted, however, in one study (56) which reported overall increased percent CRs in schizophrenia vs. controls, that when the auditory and visual EBC results are considered separately, schizophrenia patients yielded fewer CRs when the CS was an auditory vs. visual stimulus.

CR Onset Latency

One study reported shorter CR onset latencies in individuals with schizophrenia vs. controls (61). Two studies reported longer CR onset latencies in schizophrenia vs. controls (60, 64). Two studies reported no significant differences between groups (66, 67). One study reported blink onset latency results regardless of CR or UR performance, and therefore cannot be considered with either CR or UR results [see Ref. (57) in **Table 5** for these and CS-alone latency findings].

CR Peak Latency

Three studies reported shorter peak latency in individuals with schizophrenia vs. controls (61, 63, 66). One study reported longer CR peak latency in schizophrenia vs. controls (60), and three studies reported no significant differences between groups (62, 64, 65).

CR Amplitude

Five studies reported no significant differences between groups for CR peak amplitude (60, 61, 63, 66, 67). Sears and colleagues (57) reported increased CR amplitude in individuals with schizophrenia vs. controls in CS-alone trials. In *post hoc* analyses of individual blocks, Forsyth and colleagues (65) found increased CR amplitudes in controls vs. schizophrenia and SPD in later but not earlier blocks of conditioning.

Medication Effects

Of the 15 published studies, 13 reported medication status and all but one of these (56) included information specific to antipsychotic medication status. In 10 of these 12 studies, most participants in the schizophrenia sample were currently taking antipsychotic medication. In terms of conditioning effects, 8 of these 10 studies of medicated individuals reported decreased conditioning (e.g., decreased percent CRs) in individuals with schizophrenia compared to controls (58, 61–65, 67, 68). In the other two studies of medicated individuals, no group differences in conditioning rates were found (59, 60).

In 2 of the 12 studies, the entire schizophrenia group was antipsychotic-free for 3 weeks (57, 66). Sears and colleagues (57) reported facilitated conditioning in these participants, whereas Parker and colleagues (66) reported impaired conditioning. In addition, 3 of the 12 studies analyzed data from antipsychotic-free subsamples of individuals with schizophrenia (63, 64, 68). When Bolbecker and colleagues (63) re-analyzed their data including only the medication-free subset of individuals with schizophrenia and their age-matched controls (with a sample size in each group of $n = 13$, similar to other stand-alone studies of antipsychotic-free schizophrenia), they found decreased CRs and shorter CR peak latencies in these individuals with schizophrenia – with even larger effect sizes than in the full sample of individuals with schizophrenia. The authors reported no significant correlations between EBC dependent variables and chlorpromazine equivalent dosages (63), as did Brown and colleagues (61). Similarly, in a later study, Bolbecker and colleagues (64) reported no significant differences between schizophrenia participants medicated with antipsychotics vs. those who were medication-free. Finally, Coesmans and colleagues (68) reported no effect of group on percent CRs or "learning index" (change in number of CRs from first to last conditioning block) when comparing the three subgroups of individuals with schizophrenia (those taking atypical antipsychotics, typical antipsychotics, and those who were antipsychotic medication-free), and no significant correlation between learning index and chlorpromazine equivalent dosages.

Finally, both studies including intermediate schizophrenia spectrum participants [individuals with SPD (65) and first-degree relatives (67)] reported that there was no antipsychotic use in either of these populations. In these studies both individuals with SPD and first-degree relatives of individuals with schizophrenia were impaired in EBC.

Unconditioned Responses

UR measures on paired trials are reported less frequently in the literature. With regard to percentage of URs, one study reported decreased percent URs in individuals with schizophrenia vs. controls (60). With regard to UR latency, two studies reported slower UR peak latency in individuals with schizophrenia vs.

TABLE 1 | Sample characteristics for studies of EBC in schizophrenia.

Study	Samples			Diagnosis	Age matched?	Antipsychotic medication status (SZ spectrum groups)[a]
	N	Age	% Male			
Taylor and Spence (54)	42	N/A	N/A	"Psychotic"	No	N/A
	74	N/A	N/A	"Neurotic"		
O'Connor and Rawnsley (55)	20	47.2 (4.94)	100	Paranoid SZ	No	N/A
	20	41.5 (5.84)	100	Non-paranoid SZ		
	20	39.4 (12.51)	100	Control		
Spain (56)	54	40.6	59.3	Schizophrenia	No	All but 10 "were receiving some form of drug treatment"
	24	N/A	50	Control		
Sears et al. (57)	15	32.8 (9.8)	73.3	DSM-IV schizophrenia	No	Unmedicated for 3 weeks
	15	31.3 (7.2)	73.3	Control		
Hofer et al. (58)	24	30.3 (9.0)	87.5	DSM-IV schizophrenia	Yes	18 participants on atypical antipsychotics, 6 on typical antipsychotics
	20	30.9 (8.9)	85	Control		
Stevens et al. (59)	25	28.8 (6.5)	56	DSM-IV schizophrenia	Yes	Treated $\geq$14 days with stable dose of olanzapine
	25	31.1 (6.8)	56	DSM-IV schizophrenia		Taking stable dose of "classic neuroleptics" $\geq$14 days
	25	27.3 (5.6)	52	Control		
Marenco et al. (60)						
Trace	10	31.8 (8.7)	N/A	DSM-IV schizophrenia or schizoaffective disorder	Yes ($\pm$3 years)	2 participants unmedicated for $\geq$3 weeks; others taking antipsychotics
	10	33.7 (7.7)	N/A	Control		
Delay	10	41.8 (9.7)	N/A	DSM-IV schizophrenia or schizoaffective disorder	Yes ($\pm$3 years)	3 participants unmedicated for $\geq$3 weeks; others taking antipsychotics
	10	41.9 (9.4)	N/A	Control		
Brown et al. (61)	13	42 (9.56)	53.8	DSM-IV schizophrenia or schizoaffective disorder	Yes	9 participants on atypical antipsychotics, 1 on typical, 3 on both
	13	40.2 (9.0)	53.8	Control		
Edwards et al. (62)	10	40 (6.77)	60	DSM-IV schizophrenia	Yes	1 participant taking typical antipsychotics, 2 taking atypical antipsychotics, 1 taking both; 5 participants not taking antipsychotics
	8	43.5 (6.2)	62.5	Control		
Bolbecker et al. (63)	62	39.8 (9.54)	62.9	DSM-IV schizophrenia	Yes ($\pm$2 years)	12 participants unmedicated, 34 on atypical antipsychotics, 7 on typical, 9 on both
	62	39.9 (9.99)	48.4	Control		
Bolbecker et al. (64)	55	41.1 (11.1)	60	DSM-IV schizophrenia spectrum disorders	Yes ($\pm$2 years)	13 unmedicated, 36 taking atypical antipsychotics, 16 taking typical antipsychotics
	55	40.9 (11.3)	47.3	Control		
Forsyth et al. (65)	18	37.7 (9.43)	55.6	DSM-IV schizophrenia	Yes	3 SZ not taking medication at time of testing, 14 on antipsychotics; 3 SPD taking antidepressants, others unmedicated
	18	38.1 (9.87)	55.6	DSM-IV SPD		
	18	37.9 (9.85)	55.6	Control		
Parker et al. (66)	20	28.2 (9.24)	61.1	DSM-IV schizophrenia	No	5 neuroleptic-naïve, all others medication-free for 3 weeks
	20	29.2 (9.22)	50	Control		
Bolbecker et al. (67)	18	36 (12)	72.2	DSM-IV schizophrenia or schizoaffective disorder	Yes ($\pm$3 years) for each triad	2 SZ unmedicated, 13 taking atypical antipsychotics, 3 taking typical antipsychotics; 2 relatives taking antidepressants, others unmedicated
	18	35.9 (13)	38.9	Confirmed first-degree relative		
	18	36.8 (13)	44.4	Control		
Coesmans et al. (68)	38	23.9 (range = 18–35)	100	DSM-IV schizophrenia	Yes	13 antipsychotic-free (6 antipsychotic-naïve, 7 antipsychotic-free for $\geq$4 weeks), 9 taking atypical antipsychotics, 16 taking typical antipsychotics
	26	24.6 (range = 18–31)	N/A	Control		

[a] *Given the relevance of antipsychotic medication to motor abnormalities, we report here antipsychotic medication status specifically and not other psychotropic medications, except in the case of intermediate spectrum participants.*

TABLE 2 | EBC paradigms and measurement techniques for studies of EBC in schizophrenia.

Study	Procedure type	Method of eyeblink activity measurement
Taylor and Spence (54)	Single-cue visual delay EBC	Microtorque potentiometer mounted to lever attached to upper eyelid and polygraph recording eyelid position (69)
O'Connor and Rawnsley (55)	Single-cue auditory delay EBC	Light source and photoelectric cell
Spain (56)	Combined visual and auditory delay (presented as 50 trials of each, order counterbalanced)	
Auditory	Auditory delay EBC	Electrodes placed above and below the eye; similar to electrooculography
Visual	Visual delay EBC	Electrodes placed above and below the eye; similar to electrooculography
Sears et al. (57)	Single-cue auditory delay EBC	Infrared photobeam
Hofer et al. (58)	Delay eyelid conditional discrimination learning	Photocell that measured "area of the eye covered by the eyelid" (via "light reflected from the cornea")
Stevens et al. (59)	Discrimination auditory delay EBC	–
Marenco et al. (60)		
Trace	Single-cue auditory trace EBC	"Potentiometer attached to the eyelid"
Delay	Single-cue auditory delay EBC	"Potentiometer attached to the eyelid"
Brown et al. (61)	Single-cue auditory delay EBC	EMG electrodes
Edwards et al. (62)	Single-cue auditory delay EBC	EMG electrodes
Bolbecker et al. (63)	Single-cue auditory delay EBC	EMG electrodes
Bolbecker et al. (64)	ISI-shift single-cue auditory delay EBC	EMG electrodes
Forsyth et al. (65)	Single-cue auditory delay EBC	EMG electrodes
Parker et al. (66)	Single-cue auditory delay EBC	Infrared photo beam
Bolbecker et al. (67)	Single-cue auditory delay EBC	EMG electrodes
Coesmans et al. (68)	Single-cue auditory delay EBC	Magnetic distance measurement technique (i.e., measures distance between upper eyelid and lower eyelid)

controls (63, 64), while three other studies reported no significant differences between groups (61, 65, 67). Finally, with regard to UR amplitude, three studies reported increased UR amplitude in schizophrenia vs. controls (63, 65, 67), whereas three studies reported no significant group difference (61, 62, 64). And, one study reported a significant group by block interaction showing consistently diminished UR amplitude in individuals with schizophrenia compared to controls, and larger initial UR amplitude in controls that decreased across blocks (60).

Importantly, several studies explored group differences in URs to unpaired unconditioned stimuli during pre-conditioning trials or pseudoconditioning (prior to paired trial presentation). Such pre-conditioning measures test for pre-existing differences between groups in the ability to generate a blink in the absence of recent associatively salient stimuli and habituation. Marenco and colleagues (60) reported no group differences in baseline UR activity; Edwards and colleagues (62) reported no group difference in baseline UR amplitude. Bolbecker and colleagues reported no group differences in UR peak amplitude or latency in individuals with schizophrenia compared to controls in one article (67) and increased UR amplitude in another (63) – in both cases suggesting that conditioning deficits could not be accounted for by pre-existing group differences in eyeblink responses. However, Sears and colleagues (57) reported longer UR latency in individuals with schizophrenia compared to controls for US-alone trials.

Extinction

Four studies reported no significant differences between extinction rate in individuals with schizophrenia and controls (60, 61, 63, 66). However, interpretation of this finding is complicated by the group differences in percent CRs during the acquisition phase reported by three of the studies (61, 63, 66). Finally, Brown and colleagues (61) reported shorter CR onset and peak latency in individuals with schizophrenia vs. controls during extinction.

Spontaneous Blink Rate

Several studies excluded individual trials in which a blink occurred at a time during a trial that would render CR production impossible (i.e., immediately prior to CS onset) [Ref. (61–65, 67); see Ref. (60) for a more liberal window for trial exclusion]. Most of these studies also reported no significant group differences in this rough estimate of spontaneous blink rate [Ref. (60, 63–65, 67), but see Ref. (61)].

Alpha Responses

Three studies examined group differences in alpha responses, which are reflexive orienting responses to the tone (importantly, alpha responses are non-associative). All three studies reported no group differences in the rate of alpha responses (57, 58, 60). Marenco and colleagues (60) reported earlier onset of the alpha response in controls vs. individuals with schizophrenia.

EBC Correlates

Symptoms and Demographic Variables

Multiple studies have failed to find significant relationships between schizophrenia symptom severity and EBC dependent variables (61, 63, 68). Brown and colleagues (61) and Bolbecker and colleagues (63) also reported null results between symptom severity and extinction dependent variables. Parker and colleagues (66) found no significant correlations between positive or negative symptoms and the three phases of conditioning the authors used

TABLE 3 | EBC stimulus properties for studies of EBC in schizophrenia.

Study	CS properties									US properties		
	Auditory					Visual				US intensity (psi)	US intensity measurement location	Dur. (ms)
	Modality	Freq. (Hz)	Intensity	CS intensity measurement	Dur. (ms)	Modality	Intensity	CS intensity measurement	Dur. (ms)			
Taylor and Spence (54)	–	–	–	–	–	6 cm milk glass disk	24	Apparent foot-candles	520	30 mmHg = 0.58 psi	Mercury manometer	–
O'Connor and Rawnsley (55)	Tone	1100	65	dB above threshold for each subject	800	–	–	–	–	65 mmHg = 1.26 psi	N/A	500
Spain (56)												
Auditory	Tone	1000	60	"Decibels in loudness"	1000	–	–	–	–	1 g	Measured at eye	160
Visual	–	–	–	–	–	Milk glass disk	700	Millilamberts	1000	1 g	Measured at eye	160
Sears et al. (57)	Tone	1000	75	dB	500	–	–	–	–	5	N/A	100
Hofer et al. (58)	Tone	1000	65	dB SPL	800	–	–	–	–	4	N/A	80
Stevens et al. (59)	Tone	300	80	dB SPL	800	–	–	–	–	2 bar = 29 psi	N/A	100
Marenco et al. (60)												
Trace	Tone	1000	80	dB	500	–	–	–	–	Between 5 and 6	N/A	100
Delay	Tone	1000	80	dB	500	–	–	–	–	Between 5 and 6	N/A	100
Brown et al. (61)	Tone	1000	80	dB SPL	400	–	–	–	–	10	At the source	50
Edwards et al. (62)	Tone	1000	80	dB SPL	400	–	–	–	–	10	At the source	50
Bolbecker et al. (63)	Tone	1000	80	dB SPL	400	–	–	–	–	10	At the source	50
Bolbecker et al. (64)	Tone	1000	80	dB SPL	300; 400; 600; 900	–	–	–	–	10	At the source	50
Forsyth et al. (65)	Tone	1000	80	dB SPL	400	–	–	–	–	10	At the source	50
Parker et al. (66)	Tone	1000	75	dB	500	–	–	–	–	5	N/A	100
Bolbecker et al. (67)	Tone	1000	80	dB SPL	400	–	–	–	–	10	At the source	50
Coesmans et al. (68)	Tone	650	75	dB	520	–	–	–	–	Adapted to minimum intensity required to reliably evoke a UR	N/A	20

TABLE 4 | EBC experiment and analysis parameters for studies of EBC in schizophrenia.

Study	Experiment parameters								Dependent variable quantification criteria	
	ISI (ms)	Mean ITI (s)	Number of blocks	Trials per block	Total trials (no. of blocks)	Total no. paired trials	Pre-conditioning trials	Extinction trials	CR criterion (amplitude)	CR window (post-CS latency ms)
Taylor and Spence (54)	470	20	1	80	80 (1)	80	3 CS-alone, 1 US-alone	–	≥1 mm deflection of eyelid closure movement on polygraph (70)	200–470
O'Connor and Rawnsley (55)	350	Random b/t 20 and 40	1	48	48 (1)	30	3 CS-alone, 3 US-alone, 3 CS-alone	–	On CS-alone trials ($n = 18$) only: response amplitude 150% of maximum baseline amplitude (when cue light turned on before trial, 3–7 s pre-CS)	0–1.25 s post-CS onset for CS-alone test trials only
Spain (56)										
Auditory	500	20 ms	1	50	50 (1)	50	–	–	Non-voluntary eyelid movement	200–500
Visual	500	20 ms	1	50	50 (1)	50	–	–	Non-voluntary eyelid movement	200–500
Sears et al. (57)	400	12	1	70	70 (1)	63	10 US-alone trials	40 unpaired CS or US trials	Amplitude exceeds 10% of baseline UR amplitude (measured from 10 US-alone trials pre-conditioning) for paired and CS-alone trials	200–400
Hofer et al. (58)	720	12	6	12	72 (6)	48	–	–	Change in the curve of eyelid data waveform exceeds 0.4 cm for at least 30 ms on S+ and S− trials (71)	390–720
Stevens et al. (59)	700	1100 ms	1	70	70 (1)	–	–	–	–	Eyeblink response must occur 200–700 post-CS onset on S+ and S− trials
Marenco et al. (60)										
Trace	1540	18	7	11	77 (7)	70	10 CS-alone and 10 US-alone randomly presented	10 CS-alone and 10 US-alone randomly presented	Eyelid movement ≥0.5 mm for paired and CS-alone trials	150–1540
Delay	440	18	7	11	77 (7)	70	10 CS-alone and 10 US-alone randomly presented	10 CS-alone and 10 US-alone randomly presented	Eyelid movement ≥0.5 mm for paired and CS-alone trials	150–440
Brown et al. (61)	350	15	10	10	100 (10)	90	8 US-alone trials	20 CS-alone and 20 US-alone randomly presented	EMG amplitude over 5 SDs above pre-CS 125 ms baseline for each paired trial	100–350
Edwards et al. (62)	350	15	10	10	100 (10)	90	8 US-alone trials	–	EMG amplitude over 5 SDs above pre-CS 225 ms baseline for each paired trial	100–350
Bolbecker et al. (63)	350	15	10	10	100 (10)	90	8 US-alone trials	25 CS-alone and 25 US-alone randomly presented	EMG amplitude over 5 SDs above pre-CS 125 ms baseline for each paired trial	100–350
Bolbecker et al. (64)	250; 350; 550; 850	15	For each ISI: 5 (total = 10)	20	For each ISI: 100 (5) [total = 200 (10)]	For each ISI: 90 (total = 180)	8 US-alone trials	–	EMG amplitude over 5 SDs above pre-CS 125 ms baseline for each paired trial	150 pre-US onset
Forsyth et al. (65)	350	15	10	10	100 (10)	90	8 US-alone trials	–	EMG amplitude over 5 SDs above pre-CS 125 ms baseline for each paired trial	100–350

(Continued)

TABLE 4 | Continued

Study	Experiment parameters							Dependent variable quantification criteria	
	ISI (ms)	Mean ITI (s)	Number of blocks	Trials per block	Total trials (no. of blocks)	Total no. paired trials	Pre-conditioning trials	Extinction trials	CR criterion (amplitude)	CR window (post-CS latency ms)
Parker et al. (66)	400	12	3	15	45 (3)	45	10 (5 CS-alone trials and 5 US-alone trials)	3 blocks of 30 extinction trials, each consisting of 20 CS-alone trials followed by 5 CS-alone and 5 US-alone trials presented pseudorandomly	Eyeblink amplitude exceeding 10% of baseline UR amplitude (measured during pseudoconditioning US-alone trials)	200–400
Bolbecker et al. (67)	350	15	10	10	100 (10)	90	8 US-alone trials	–	EMG amplitude over 5 SDs above pre-CS 125 ms baseline for each paired trial	100–350
Coesmans et al. (68)	500	Random intervals b/t 20 and 30	10	8	80 (10)	60	–	–	For paired and CS-alone trials, maximum eyelid closure occurring between 100 and 800 ms post-CS onset was first calculated. Blinks then classified as CRs if blink onset occurred within CR window (72)	150–525

to analyze their EBC data (i.e., early, middle, and late); however, negative symptoms were significantly correlated with late-phase extinction of the CR. In the earliest examination of symptom correlates of EBC, O'Connor and Rawnsley (55) reported no significant correlation between EBC and introversion scores [but see Spain (56) for EBC correlates of clinician-rated withdrawal]. Finally, in their investigation of demographic correlates of EBC, Coesmans and colleagues (68) also reported non-significant correlations between learning index and age and years of education.

Neuropsychological Variables

Bolbecker and colleagues (63) reported significant positive correlations between average percent CRs and both WASI IQ estimates and the WASI Vocabulary subscale in controls, but not in individuals with schizophrenia. The Matrix Reasoning subscale was not significantly correlated with average percent CRs in either group. Forsyth and colleagues (65) reported a significant positive correlation between percent CRs and Digit Symbol score (a subscale of the WAIS) for schizophrenia spectrum participants (i.e., individuals with schizophrenia and SPD were combined into one group). This significant correlation held when individuals with schizophrenia were analyzed separately, but not when individuals with SPD were analyzed separately. Additionally, the authors reported no significant correlations between Digit Symbol score and percent CRs in controls, or between percent CRs and the Picture Completion, Similarities, or Digit Span WAIS subscales in either controls or schizophrenia spectrum participants (65). Using aggregate cognitive domain scores from a battery of neuropsychological tests in patients, Parker and colleagues (66) reported a significant positive relationship between both aggregate language and motor scores and CR timing during early conditioning; motor scores were also correlated with middle-phase extinction of the CR. Finally, Coesmans and colleagues (68) reported a significant positive correlation between EBC learning index and saccade adaptation strength in controls, but not individuals with schizophrenia, while no significant correlations were found in either group between EBC learning index and saccade adaptation speed.

Neuroimaging Measures

In a study of cerebellar volumetric correlates of EBC, Edwards and colleagues (62) reported a significant positive correlation between anterior lobe volume and CR onset latency, and a significant negative correlation between anterior lobe volume and UR amplitude (in response to paired trials) in controls, but no significant correlations between cerebellar MRI volume and EBC dependent variables in individuals with schizophrenia. Parker and colleagues (66) analyzed PET data according to phases of conditioning (i.e., early, middle, and late), and reported decreased rCBF in individuals with schizophrenia compared to controls in frontal, thalamic, and cerebellar regions during both acquisition and extinction (among other loci). In summarizing findings of hypofrontality during EBC, the authors highlighted decreased rCBF in individuals with schizophrenia compared to controls in the contralateral medial frontal gyrus during all phases of conditioning, and the contralateral middle frontal gyrus during the early and middle phases of conditioning. The authors also highlighted decreased rCBF in contralateral cerebellar lobules IV and V in

TABLE 5 | Summary of main findings from studies of EBC in schizophrenia.

Study	Summary of major findings
Taylor and Spence (54)	*CR* – Trend for increased percent visual CRs in "psychotics" vs. "neurotics."
O'Connor and Rawnsley (55)	*CR* – No significant difference in number of CRs in response to CS-alone trials between groups (chronic paranoid SZ, chronic non-paranoid SZ, control). *Extinction* – Chronic paranoid SZ had significantly smaller "extinction scores" than controls.
Spain (56)	
Auditory	*CR* – Increased overall number of CRs in SZ vs. control, but effect not significant when examining subgroups matched for skin potential. SZ had significantly more visual than auditory CRs; opposite relationship in HNs. CRs for auditory EBC fewer in SZ vs. HN (but no statistical test reported).
Visual	*CR* – Increased overall number of CRs in SZ vs. control, but effect not significant when examining subgroups matched for skin potential. SZ had significantly more visual than auditory CRs; opposite relationship in HNs. CRs for visual EBC greater in SZ vs. HN (but no statistical test reported).
Sears et al. (57)	*CR* – SZ had significantly higher %CRs than controls and reached 70% CR learning criterion significantly faster (i.e., earlier in the experiment) than controls. Significantly shorter onset latency of all blinks in SZ vs. controls during paired trials (however, difference is not significant when group differences in conditioning level were accounted for and for CS-alone trials). CR amplitude significantly increased in SZ vs. controls in CS-alone trials. *UR* – Significantly longer UR latency in SZ vs. controls on US-alone trials.
Hofer et al. (58)	*CR* – Trend for controls to develop first CR before SZ. No significant difference between S+ and S− in SZ; there was a significant difference in controls for increased CRs to S+ vs. S−. Significantly greater %CRs in controls vs. SZ for S+ but no significant difference for S−. Significant group x reinforcement type (S+ or S−) x block interaction indicated controls showed increased %CRs in response to S+ as the experiment progressed.
Stevens et al. (59)	*CR* – No significant differences between groups in number of trials to reach learning "criterion" (i.e., 5 consecutive trials with an eyeblink response $<$500 ms pre-US onset to S+ but not S−).
Marenco et al. (60)	
Trace	*CR* – Analysis using the entire CR window appeared to be contaminated by spontaneous blinks (especially in SZ). A second analysis examining when in the CR window responses occurred revealed that SZ demonstrated increased early conditioned responses vs. controls, and slightly fewer later responses vs. controls. Frequency of early responses did not increase over time for SZ; control participants demonstrated trend-level increases in early responses over time. No significant effects when examining the last 500 ms as the CR window.
Delay	*CR* – No group differences in %CRs. Longer CR onset and peak latency for SZ vs. controls in "conditioners" during paired trials and CS-alone trials. More efficient "workratio" (a measure of CR efficiency of closing the eye at the time of US onset) in SZ vs. control "conditioners" during paired trials and CS-alone trials. *UR* – %URs significantly lower in SZ vs. controls in entire sample during paired trials. UR amplitude did not decrease across blocks in SZ vs. control "conditioners" during paired trials. For CS-alone trials, %UR-range responses significantly decreased in SZ vs. controls for entire sample (even larger effect when examining "conditioners" only).
Brown et al. (61)	*CR* – Significantly fewer %CRs overall in SZ vs. controls, and a trend for controls acquiring more CRs over time than SZ. Significantly shorter CR onset and peak latency in SZ vs. controls. Controls demonstrated decreased CR onset variability over time; SZ did not. *UR* – Trend for longer UR peak latency in SZ vs. control. *Extinction* – Significantly shorter CR onset and peak latency for SZ vs. controls.
Edwards et al. (62)	*CR* – Marginally significant difference between groups in learning, as indexed by the difference between mean %CRs in the last two blocks and mean %CRs in the first two blocks. Significantly higher %CRs in controls vs. SZ in block 9 of conditioning. *UR* – No significant differences in UR peak amplitude for paired or unpaired trials. No significant correlation between unpaired UR peak amplitude and mean CR amplitude.
Bolbecker et al. (63)	*CR* – Significantly decreased %CRs and shorter CR peak latency in SZ vs. controls. *UR* – Significantly slower UR peak latency in SZ vs. controls during paired trials. Significantly higher UR peak amplitude in SZ vs. controls for paired and unpaired trials. *Extinction* – Trend for fewer CRs during extinction for SZ vs. controls.
Bolbecker et al. (64)	*CR* – Decreased %CRs in SZ vs. controls across ISIs and later (i.e., closer to US) CR onset latency in SZ vs. controls across ISIs. *UR* – Significantly shorter UR latency in controls vs. SZ when first ISI presentation examined only (effect not significant when both first and second ISI presentations are considered).
Forsyth et al. (65)	*CR* – Decreased %CRs in SZ and SPD vs. controls, specifically in later blocks of conditioning. Trend for shorter CR peak latency in SZ and SPD vs. controls. CR amplitudes larger in a few later blocks in controls vs. SPD and SZ. *UR* – Significantly higher UR peak amplitude in SZ vs. controls and SPD.
Parker et al. (66)	*CR* – Significantly greater %CRs in controls compared to SZ in middle and late phases of conditioning. CR peak latency significantly shorter in SZ in middle phase of conditioning.
Bolbecker et al. (67)	*CR* – Significantly lower rate of learning in SZ and relatives compared to controls. Controls increase in %CRs over time more than relatives and SZ. *UR* – Larger UR amplitude during paired trials only in SZ vs. controls.
Coesmans et al. (68)	*CR* – Significantly fewer %CRs in SZ compared to controls, with a trend-level group x block interaction. Controls demonstrated significantly higher learning index (defined as the difference in first and last block number of CRs) vs. SZ.

SZ, individuals with schizophrenia; HN, healthy non-psychiatric controls; SPD, individuals with schizotypal personality disorder.

individuals with schizophrenia compared to controls during all phases of conditioning, with a group difference in ipsilateral cerebellar lobule VI during late acquisition only. Finally, group differences in rCBF in the thalamus were significant during early and late conditioning. Regarding rCBF during extinction, the authors highlighted decreased rCBF in individuals with schizophrenia compared to controls during all phases of extinction in the medial and middle frontal gyri and in cerebellar lobule IX. Additional loci of decreased cerebellar rCBF in individuals with schizophrenia compared to controls included cerebellar lobules IV and V during middle extinction, and cerebellar lobules IV, V, and VI during late extinction. Finally, the authors highlighted that decreased thalamic rCBF in individuals with schizophrenia was significant during early phase extinction (66).

DISCUSSION

Conditioning (i.e., %CRs)

In reviewing the literature investigating delay EBC in schizophrenia, decreased percent conditioned responses in individuals with schizophrenia compared to non-psychiatric controls emerges as the single consistent, robust, and replicated finding. Diminished conditioning in schizophrenia is highly suggestive of cerebellar dysfunction, given the crucial role of the cerebellum in the circuit underlying delay EBC. Moreover, as discussed in the following paragraphs, there are no extraneous variables (i.e., medication status, sample size, different analytical approaches, parametric variability, non-associative blinking function, and investigative group) that could fully account for these EBC deficits in schizophrenia.

In investigating the possible driving role of medication in the observed EBC deficits (i.e., decreased %CRs) in individuals with schizophrenia, it is crucial to note that both medicated and non-medicated samples demonstrate conditioning deficits in individuals with schizophrenia (see Medication Effects subsection of Section "RESULTS"). Also important to this question of the effect of antipsychotic medication on EBC are the findings of EBC deficits in a non-medicated subsample (63), as well as the failure to find group differences in medicated vs. unmedicated individuals with schizophrenia (64, 68).

However, it is important to note that even the "medication-free" samples and subsamples reported above are not medication-naïve samples. While a small number of participants in the most recent studies [$n = 5$ in Parker et al. (66), and $n = 6$ in Coesmans et al. (68)] were naïve to antipsychotics, the small sizes of these groups precluded meaningful analyses investigating the effect of antipsychotic-naïve medication status. Therefore, while it appears unlikely based on the current review that recent use of antipsychotic medication drives EBC deficits, it is impossible to rule-out the long-term effects of antipsychotic use in individuals with schizophrenia in the results of the study of "medication-free" samples and subsamples.

Eyeblink conditioning studies of intermediate genotypes and phenotypes of schizophrenia such as first-degree relatives (67) and SPD (65) that have demonstrated conditioning deficits in these groups are very important, especially given the absence of studies using medication-naïve or first-episode schizophrenia groups. Neither of these study groups were taking antipsychotic medication. This suggests that EBC deficits are related to the genetic/biological pathophysiology of schizophrenia, not the history of or current antipsychotic medication use.

In addition to medication status, examination of **Tables 1–5** reveals no systematic sample characteristic, parameter, or analytic approach that could be driving this review's main finding of EBC deficits in schizophrenia. Indeed, EBC deficits occur across samples of varying ages and gender composition, and in studies using a range of EBC stimulus parameters and experimental design (e.g., CS/US duration, ISI, ITI, and pre-conditioning trials or pseudoconditioning) and analysis (e.g., CR window and criterion) specifications. Furthermore, potentially confounding issues such as spontaneous blink rate and baseline blinking function have been investigated by several groups, with no convincing evidence that these variables bias EBC experimental results.

Furthermore, it appears as though many studies reporting null findings or facilitated conditioning may have parametric or analytic variations that could account for such results. Specifically, Taylor and Spence (54) used a visual delay EBC paradigm, and the diagnostic criteria for the disorder differed substantially from those used in recent decades. Furthermore, the idiosyncratic analytic approaches of other studies may account for the reported null findings. For example, rather than quantifying rate of conditioning, Stevens and colleagues (59) measured the number of trials it took for participants to reach "criterion," or five consecutive CRs. This style of analysis is not reported in most other studies. Another study appeared to restrict their analysis such that relatively less data are included compared to other studies. Specifically, O'Connor and Rawnsley (55) only used 18 unpaired CS-alone trials to measure conditioning, rather than attempting to detect CRs across all paired trials over the course of conditioning. Finally, Sears and colleagues (57) did not include a measure of spontaneous blink rate; it is therefore possible that group differences in non-associative blinking could have confounded the reported findings of facilitated conditioning in schizophrenia. More research is necessary to determine whether these varied findings are due to these methodological differences or, in fact, reflect inconsistencies in EBC deficits in schizophrenia across studies.

CR Timing

Group differences in timing of the conditioned response (i.e., onset and peak latency) have been reported far less frequently than rate of conditioning (i.e., percent CRs). Among studies reporting these variables, there is inconsistency in how onset latency is calculated and whether the algorithm used to calculate onset latency is reported. Results are also inconsistent, with findings reported in both directions and null results. However, the proportion of findings reporting some group difference ($N = 7$) in CR timing vs. null results ($N = 6$) suggests that there may be abnormalities in the timing of the conditioned response in individuals with schizophrenia.

Interpretation of Correlate Findings

Parker and colleagues' (66) findings of both impaired conditioning and decreased cerebellar blood flow in individuals with

schizophrenia compared to controls during delay EBC strongly suggest that cerebellar neural dysfunction underlies the behavioral EBC abnormalities consistently reported in individuals with schizophrenia. This is a crucial piece of evidence, as authors reporting previous findings of impaired delay EBC in individuals with schizophrenia have inferred underlying cerebellar dysfunction given the well-established delay EBC cerebellar circuitry in non-human animals.

In addition, EBC correlates of neuropsychological performance are reported by a few studies (63, 65, 66). This shared variance between cerebellar-dependent EBC performance and cognition indicates that the cerebellum may be a shared neural substrate between these two processes, which is consistent with cerebellar involvement in cognitive as well as motor function.

Limitations and Future Directions

One critical conclusion from this review is that antipsychotic medications do not appear to be driving the EBC deficit observed consistently in schizophrenia. However, this conclusion is primarily based on the study of EBC in unmedicated (rather than never-medicated) individuals with schizophrenia, first-degree relatives and individuals with schizotypal personality disorder. While the robustness of the EBC deficit in these populations is obviously compelling, a logical and important next step is conducting delay EBC in first episode and/or never-medicated individuals. Second, significant variability in methodological and analytic strategies across EBC studies precluded a meta-analytic approach; therefore, as more studies are conducted using consistent methods, statistical analyses, and reporting, this approach should be considered. Third, further replication of the main findings of this review article (i.e., an EBC deficit in schizophrenia) is essential given that one investigative group has accounted for most patients studied (6 of 15 studies).

Finally, further work investigating the neural activity in the cerebellum during delay EBC in schizophrenia is essential to elucidating the specific contribution of the cerebellum in driving impairments in delay EBC. Specifically, the fine-grained spatial resolution of fMRI could prove essential to understanding which regions of the cerebellum underlie delay EBC in humans, and where this circuit is degraded in schizophrenia.

EBC Findings Within the Context of Theories of Schizophrenia

Overall, the reported deficits in cerebellar-dependent EBC in individuals with schizophrenia are consistent with the theory of cognitive dysmetria, in which the cerebellum is one node in a circuit regulating the fluid temporal coordination of motor, cognitive, and affective information, the disruption of which is hypothesized to be a common underlying precursor to the heterogeneous downstream expressions of the phenomenology of schizophrenia (29, 73). The cerebellum is believed to play a unique role in this circuit mediating the coordination (or instantiating the discoordination) of mental activity, which also includes the prefrontal cortex and the thalamus. Specifically, it is the feedback (via the thalamus) between the prefrontal cortex (and the higher-order cognitive processes instantiated therein) and the cerebellum (which is notable for cytoarchitecture conducive to large-scale parallel processing and its role in coordination, sequencing, and timing) that is hypothesized to instantiate the fluid temporal coordination of mental activity (29, 73). As stated above, consistent deficits in performance on one of the most robust and well-understood (with respect to underlying circuitry) cerebellar tasks in individuals with schizophrenia provide evidence consistent with the theory of cognitive dysmetria, and this finding is germane to its arguably most critical node [Andreasen (29) initially identified assays of cerebellar function, specifically citing EBC as a potential example, as the litmus test through which the theory can be falsified]. In addition, the relationship between cerebellar function and cognitive function supports this theory (63, 65, 66).

More specifically, the possible mechanisms of the cerebellum's contribution to higher-order cognitive function have been hypothesized to parallel that proposed by control theory in the domain of motor control [see Ref. (74, 75), for review]. The cerebellum is hypothesized to contribute to the coordination of movement via internal models (both forward and reverse) (74), which are neural representations that can be trained to simulate the dynamics of motor action (74–76). Forward models are believed to receive input that duplicates the motor command (termed an efference copy) sent by the motor cortex (which controls movement) after the motor cortex receives a higher-order instructor command (i.e., from the premotor cortex), and output a prediction of what the sensory consequences of that command will be (a corollary discharge). The forward model is tuned by a mechanism that compares sensory predictions of the model to actual sensory input, which has been hypothesized to occur in the inferior olive. Once a forward model is adequately trained, it can provide useful feedback (via the thalamus) to the primary motor cortex, which executes motor commands (74, 75). An inverse model, conversely, can eventually conduct feed-forward motor control in response to a higher-order instructor command. While error-related feedback processing in the inferior olive is also hypothesized to tune inverse models, inverse models are trained by comparing motor output to the initial instructor command, and this feedback is mediated through the motor cortex (74).

In generalizing the function of internal models in the cerebellum to a role in cognition, it has been proposed that there are areas in the prefrontal cortex (following a higher-order instructor command, as in the example using motor function) that send commands to areas of the cortex that instantiate psychological processes and manipulate these areas in much the same way the motor cortex manipulates the motor system. In this way, the cerebellum receives an efference copy of this command and can learn and execute forward models that would simulate processing in the target brain area and provide feedback to the prefrontal cortex (74, 75). Using an inverse model, the cerebellum could actually perform feed-forward control of cognitive function (again following an instructor signal) by acting directly on the target brain area (74).

These putative mechanisms of cerebellar contributions to cognition are supported by the frequently cited uniformity of cerebellar cytoarchitecture, which, along with its circuitry suggest that the cerebellum is performing a uniform process across a variety of cortical inputs (74, 75). In addition, the matched increase in both cerebral and cerebellar neurons in humans as well as high

connectivity between the cortex and cerebellum also indicate the proposed mechanisms of cerebellar contributions to cognition are physiologically plausible (77, 78). Finally, translational evidence in support of cerebellar contributions to cognition can be found in comparing cortical projections to the cerebellum in humans and macaque monkeys, where the largest proportion of the projections in humans originates in prefrontal cortex vs. motor areas in macaque monkeys [see Ref. (75) for review]. In light of this physiological and translational evidence, the proposed function of internal models as a mechanism of cerebellar contributions to cognition seems both anatomically and evolutionarily sound. Ramnani (75) has further described internal models as ideally suited to rapid, highly accurate, efficient processing of routine, well-practiced cognitive processes, whereas cortical mechanisms are best suited for flexible though less efficient processing, which would be important for processing novel problems or generalizing cognitive processes across different contexts.

In addition to being a robust assay of cerebellar function, EBC is especially germane to the putative mechanisms outlined above in light of the proposed mechanism of error correction of internal models. Specifically, the feedback-related tuning of internal models is believed to be instantiated through error signals sent from climbing fibers (originating in the inferior olive), which results in LTD at the parallel fiber–Purkinje cell synapse when climbing and parallel fibers are simultaneously activated (74). In EBC, US information is transmitted through climbing fibers from the inferior olive, and is often conceptualized as an error signal, and an identical LTD mechanism as that described above is believed to be an integral part of cerebellar cortical plasticity during conditioning [see Ref. (79) for review]. It has previously been suggested that dysfunctional internal models may be the mechanism of cerebellar-mediated cognitive and affective dysfunction in schizophrenia (22, 74). It is therefore notable that the findings of this review indicating deficits in cerebellar function in schizophrenia, and more importantly EBC deficits specifically, may be indicative of dysfunctional cerebellar internal models, which may be mediating the cardinal cognitive and affective symptoms of the disorder.

However, neuropsychological correlates of EBC in individuals with schizophrenia have been rarely investigated. Furthermore, the consistently reported non-significant correlations between delay EBC and symptom severity in individuals with schizophrenia is surprising given the putative role of the cerebellum in the pathophysiology of schizophrenia. It is possible that the contributions of cerebellar deficits to the pathological processes of schizophrenia are more proximal effects on timing and coordination of information, whereas symptoms and impaired neuropsychological function are more distal manifestations of the disorder that are affected by many factors and are not linearly related in magnitude to cerebellar dysfunction. Restricted range in neuropsychological and symptom measures and/or floor effects might also obscure any systematic relationships between these variables and EBC performance. Finally, symptoms are a state-dependent variable; the potentially transient and fluctuating nature of symptom severity might also account for the lack of reported correlates. Alternatively, it is possible that cerebellar dysfunction in areas outside of the delay EBC circuitry is related to symptom severity and neuropsychological function. Still, more research is necessary to understand the relationships between cerebellar-mediated dysfunction and cognitive and clinical variables.

Importantly, EBC performance deficits in schizophrenia may have implications for glutamatergic models of the disorder given that glutamate is the primary excitatory neurotransmitter in the cerebellum [see Ref. (80) for review]. The glutamate model of schizophrenia hypothesizes dysfunction of the NMDA type of glutamate receptor [see Ref. (81) for overview]. Non-human animal research has implicated NMDA receptors in the interpositus nucleus in CR acquisition [Ref. (82); see Ref. (79) for a thorough review of the neural mechanisms of EBC]. Given that the "memory trace" of delay EBC has been localized to the anterior interpositus nucleus, it is therefore possible that impairments in conditioning in schizophrenia (reported most frequently as a decrease in percent CRs, or impaired CR acquisition) are related to NMDA receptor dysfunction in the interpositus nucleus in schizophrenia.

There is also substantial glutamatergic transmission in the cerebellar cortex; therefore, abnormalities in CR timing (largely mediated by the cerebellar cortex) may also be indicative of NMDA receptor dysfunction in schizophrenia. While NMDA receptors have been reported in the cerebellar cortex (80), they were traditionally not believed to play a role in the cellular mechanism (i.e., LTD at the parallel fiber-Purkinje cell synapse following both parallel and climbing fiber input to Purkinje cells) believed to underlie EBC-related learning in the cerebellar cortex [see Ref. (83) for review]. Importantly, however, there is more recent evidence that NMDA receptors at the climbing fiber–Purkinje cell synapse may in fact contribute to LTD at the parallel fiber–Purkinje cell synapse (84). Furthermore, more broad conceptualizations of the substrates of cerebellar learning are emerging that suggest that mechanisms of cerebellar cortical plasticity and neural activity beyond LTD at the parallel fiber-Purkinje cell synapse (some involving NMDA receptors) may be involved in EBC (85, 86). Accordingly, more research is necessary to determine the role of glutamate in reported EBC timing abnormalities in schizophrenia.

In addition to the glutamate hypothesis, abnormalities in the endocannabinoid system in schizophrenia [see Ref. (87) for brief review] are also implicated by the current review findings. Edwards and Skosnik (87) have proposed EBC neural circuitry including endocannabinoids as retrograde signals serving to neuromodulate cerebellar cortical activity, thereby influencing CR timing and morphology. It is therefore possible that CR timing abnormalities in schizophrenia are indicative of abnormalities in the endocannabinoid system [see Ref. (87) for discussion].

AUTHOR CONTRIBUTIONS

JK, WH, AB, and BO conceptualized the review article. JK conducted the review. JK and WH drafted the paper, and AB and BO provided critical review. All authors approved and agree to be accountable for the final version of the manuscript.

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New Insights into the Nature of Cerebellar-Dependent Eyeblink Conditioning Deficits in Schizophrenia: A Hierarchical Linear Modeling Approach

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Evidence of cerebellar dysfunction in schizophrenia has mounted over the past several decades, emerging from neuroimaging, neuropathological, and behavioral studies. Consistent with these findings, cerebellar-dependent delay eyeblink conditioning (dEBC) deficits have been identified in schizophrenia. While repeated-measures analysis of variance is traditionally used to analyze dEBC data, hierarchical linear modeling (HLM) more reliably describes change over time by accounting for the dependence in repeated-measures data. This analysis approach is well suited to dEBC data analysis because it has less restrictive assumptions and allows unequal variances. The current study examined dEBC measured with electromyography in a single-cue tone paradigm in an age-matched sample of schizophrenia participants and healthy controls (N = 56 per group) using HLM. Subjects participated in 90 trials (10 blocks) of dEBC, during which a 400 ms tone co-terminated with a 50 ms air puff delivered to the left eye. Each block also contained 1 tone-alone trial. The resulting block averages of dEBC data were fitted to a three-parameter logistic model in HLM, revealing significant differences between schizophrenia and control groups on asymptote and inflection point, but not slope. These findings suggest that while the learning rate is not significantly different compared to controls, associative learning begins to level off later and a lower ultimate level of associative learning is achieved in schizophrenia. Given the large sample size in the present study, HLM may provide a more nuanced and definitive analysis of differences between schizophrenia and controls on dEBC.

Keywords: schizophrenia, eyeblink conditioning, cerebellum, associative learning, reflex conditioning, conditioned response, cognition, psychosis

INTRODUCTION

Schizophrenia is a complex disorder with diverse symptoms and heterogeneous expression. Besides its cardinal psychotic symptoms, cognitive and motor abnormalities are prominent symptoms of the disorder. The cognitive dysmetria theory of schizophrenia (1) provides a unitary framework that can account for the disparate symptoms of schizophrenia. It posits that disruptions in the

cortico–cerebello–thalamo–cortical circuit (CCTCC) lead to poor coordination of information, resulting in different symptom constellations. Given that the cerebellum plays a role in temporal processing (2), it may occupy a unique role in this circuit by modulating the temporal coordination of information. Consistent with this proposition, evidence collected over the last several decades points to not only an important cerebellar role in coordinated movement and motor learning, but also non-motor psychological processes, most notably cognition (3–8). The neuroanatomical substrate for these functional effects has been revealed by studies confirming that the CB is reciprocally connected to prefrontal, parietal, and motor/premotor cortex (9–12). It is not surprising then that lesions to the cerebellum can produce symptoms commonly seen in schizophrenia, including visuospatial deficits, attention deficits, executive dysfunction, flattened affect, disinhibited, and socially inappropriate behavior (6).

Neuropathological and neuroimaging studies have documented morphological and functional cerebellar abnormalities in schizophrenia. For example, subjects with schizophrenia have reduced bilateral cerebellar volume (13), abnormal cerebellar connectivity to cerebral regions involved in both motor and cognitive functions (14), cerebellar morphological abnormalities (15), and reductions in Purkinje cell size and density (16–18). Even groups at clinical and familial risk for psychosis show reduced cerebellar gray matter (19) compared to non-risk groups. However, negative findings exist both in the neuroimaging (20) and neuropathology (21) literature.

Importantly, first-episode (22–24) and antipsychotic medication naïve schizophrenia patients (25) have reduced cerebellar volume, suggesting that cerebellar abnormalities are characteristic of the disorder rather than medication use. Perhaps most convincingly, cerebellar volume is associated with cognitive deficits (26) as well as symptoms of depression, negative symptoms, and psychotic features in schizophrenia (25, 27, 28), suggesting that illness severity or progression may coincide with cerebellar degradation.

Delay eyeblink conditioning (dEBC) is an associative learning task that is highly dependent upon cerebellar functioning (29–31), in. The neuro-circuitry of this task has been extensively studied, and evidence overwhelmingly supports the conclusion that the cerebellum is critical both for learning the association between the unconditioned and conditioned stimuli and for the expression of the conditioned eyeblink response (32, 33). Numerous additional brain regions (i.e., hippocampus, medial septum, frontal cortex) can change the way in which the eyeblink response is expressed (34), neuroplasticity in the cerebellum initially elicits the classically conditioned eyeblink response (35).

Over the past decade, accumulating evidence indicates that cerebellar-mediated dEBC associative learning is abnormal in schizophrenia (36–38), schizotypal personality disorder (39), and first-degree relatives of schizophrenia patients (38). These associative learning deficits in schizophrenia may be remediated by pharmacological intervention (37).

One outstanding issue in the dEBC literature is statistical in nature. Specifically, a repeated-measures analysis of variance (ANOVA) is commonly used to analyze dEBC data, despite the availability of superior and more sophisticated statistical techniques, such as hierarchical linear modeling (HLM), which may reveal more reliable and nuanced findings. In our previous studies of dEBC using ANOVA, we have found conflicting results with respect to whether the learning rate (e.g., the block by group interaction in ANOVA) differs between groups. Several studies have found that the schizophrenia group had a reduced acquisition rate (36, 39), while others found no difference between groups (38, 40). Notably, the study with the largest sample size ($N = 62$) found a reduced average percentage of conditioned responses from subjects with schizophrenia, but no between-group differences in acquisition rate compared to healthy controls (40).

Hierarchical linear modeling is particularly well suited to dEBC data analysis and is superior to repeated-measures ANOVA for measuring time-dependent change because it takes into consideration the statistical dependencies in repeated-measures designs. HLM can be considered a special case of regression that can accommodate variance on more than one level (i.e., nested data), in this case, at both the individual level and at the group level. In HLM, the best-fitting line for each individual is identified, but each line fit is also influenced by the trajectories of other group members. This aspect of HLM has the effect of increasing the accuracy of each individual's fit while minimizing the error of measurement at the individual and group level. Moreover, HLM has less restrictive assumptions, can tolerate missing data points, and can accommodate hierarchical or nested data structures (41). Perhaps the greatest strength of HLM is that heterogeneity of variance is treated as potentially meaningful information that can help to identify significant interactions between variables (42), whereas in ANOVA it is treated as a nuisance factor. Finally, HLM can be used to examine growth curves that model traditional learning curves so that important parameters, such as the slope, asymptote, and inflection point of the fitted curves can be quantified. [For a more comprehensive explanation of the use of HLM in repeated-measures designs, please see Ref. (43)].

Hierarchical linear modeling was implemented in a recent study (44, 45) in which dEBC data from healthy controls, individuals with schizophrenia, and first-degree relatives of individuals with schizophrenia ($N = 18$ per group) were fitted to a linear model. Differences in acquisition rate (i.e., slope), indicating a slower rate of associative learning was found between both the schizophrenia and family members groups compared to controls. In the present study, data from a larger schizophrenia sample was age-matched to controls ($N = 59$ per group) and HLM was applied to a three-parameter logistic growth model to more closely approximate a learning curve. We predicted that the slope of the learning curve would be lower for the schizophrenia group, indicating a slower learning rate. We also expected that the asymptote – the maximum level of performance – would be lower in schizophrenia, and that the inflection point, which is the point on the learning curve when learning begins to slow down and level off, would occur later.

MATERIALS AND METHODS

Participants

Participants were 56 individuals (17 females) who were diagnosed with schizophrenia and 56 age-matched control participants (29

females). Control participants had no history of psychotic and mood disorders and no history of schizophrenia spectrum disorders within first-degree relatives. Data from 36 individuals with schizophrenia (12 females) and 32 controls (15 females) included in this study had been included in an earlier study of dEBC that used more traditional analysis methods (40). Participants with schizophrenia were recruited through outpatient and inpatient units at local hospitals. The control group was recruited by posting community and newspaper advertisements. Participants' demographic, clinical, and medication information can be seen in **Table 1**. Welch's *t*-test showed that, as expected due to age-matching, the mean age of schizophrenia participants did not differ from controls [$t(1,112) = -0.29$, $P = 0.77$]. Sex was significantly different across groups [$\chi 2(1) = 4.46$, $P = 0.035$], with more males in the schizophrenia group (see **Table 1**). Importantly, sex was used as a covariate in the HLM analyses and it did not significantly improve model fit ($p > 0.05$).

The Diagnostic and Statistical Manual of Mental Disorders-IV Axis I Disorders (SCID-I) (46) sections for mood disorders, psychotic disorders, and substance abuse disorders was used to diagnose participants in the schizophrenia group. Medical records were consulted to refine diagnoses when necessary. The non-patient version of SCID-I (47) sections for mood, psychotic, and substance abuse, as well as the SCID II, was used to identify controls without a history of psychiatric or personality disorders. The positive and negative syndrome scale (PANSS) (48) was used to rate clinical symptoms in the schizophrenia group. A total of 53 of the 56 participants in the schizophrenia group had PANSS scores available within 2 weeks of the time of dEBC testing.

Participants were excluded from the experiment if they had clinically significant hearing loss, cardiovascular disease, an intelligence quotient (IQ) score of less than 70, had received electroconvulsive therapy, or if they had a history of neurological disorders, head injury resulting in loss of consciousness, or alcohol or substance dependence within the 3 months prior to their participation in the experiment. Additional exclusion criteria for potential control group participants were history of psychotic or mood disorders, or having a first-degree relative with a schizophrenia spectrum diagnosis. All aspects of this study were approved by the Indiana University Human Subjects Institutional Review Board (IUB-IRB; Protocol #1009001702), and all participants provided written informed consent prior to participation in the study.

TABLE 1 | Demographic, clinical, and medication information.

	Schizophrenia	Controls
Age (years)	*M* = 36.4 (SD = 10)	*M* = 35.8 (SD = 10)
Sex (M:F)	39:17	27:29
PANSS total score	*M* = 59 (SD = 13)	–
Positive	*M* = 16 (SD = 6)	–
Negative	*M* = 15 (SD = 5)	–
General	*M* = 28 (SD = 6)	–
[a]Past alcohol dependence	13	0
Past illicit drug dependence	16	0
[b]Psychotropic medication		
No antipsychotic medication	6	56
Atypical antipsychotic	44	0
Typical antipsychotic	12	0

[a]*Nine schizophrenia patients met criteria for both past alcohol and other drug dependence.*

[b]*Eight schizophrenia patients were taking both typical and atypical antipsychotic drugs at the time of testing. Medication information was not available for two participants with schizophrenia.*

Delay Eyeblink Conditioning Procedure

The experiment consisted of 10 blocks of dEBC, with 10 trials per block. Of these 10 trials, 9 were paired with a conditioned stimulus tone lasting 400 ms (1000 Hz, 80 dB) that co-terminated with a 50 ms unconditioned stimulus air puff (10 psi at the source). A single tone-alone trial was also randomly presented during each block. The experiment began with eight unconditioned stimuli (15 s average inter-trial interval with a range of 10–20 s) that were presented alone to assess the integrity of eyeblink responses. Participants rated neutral pictures from the International Affective Picture System (49) throughout the experiment to maintain alertness. Pictures were presented for 2 s between trials and participants indicated the pleasantness of each picture on a response pad. Participants were monitored using a closed circuit camera to ensure their eyes remained open during the experiment. In cases in which a participant's eyes appeared to close, the experiment was briefly suspended so alertness could be re-established by turning on the lights and offering the participant a drink of water.

Procedure

Electromyographic activity was recorded from the orbicularis palpebrarum of the left eye by placing two bipolar electrodes 1 cm below the left eyelid, approximately 1 cm apart, and centered beneath the pupil. A ground electrode was placed on the forehead. The 50 ms unconditioned stimulus air puff was delivered to the left eye via copper tubing affixed to lens-less glasses and connected to plastic tubing (approximately 120″) connected to a regulator. Ear inserts (E-A-RLINK – Aearo Company Auditory Systems) were used to deliver the conditioned stimulus tone. Electromyographic recordings were continuously recorded (2.5 kHz A/D rate; high-pass filter = 1 Hz; low-pass filter = 500 Hz; gain = 1000) and stored offline for further processing.

Data Processing

The continuous dEBC data files were segmented into 1086 ms epochs starting 500 ms before the conditioned stimulus onset. Data were high-pass filtered using a 28 Hz (6 dB per octave) filter, rectified, then smoothed using a 41 point Gaussian weighted moving average. The 90 paired dEBC trials from each experiment were analyzed using DataMunch, a MatLab program specifically designed for eyeblink conditioning data analysis (36, 38–40, 44, 45, 50–52). Blinks that occurred between 25 and 100 ms were characterized as alpha responses, which occur in response to the conditioned response tone onset and are reflexive, orienting responses that are not learning-related phenomena. For each participant, eyeblinks were counted as conditioned responses if they exceeded 5 SDs of baseline activity (baseline = 125 ms prior to conditioned stimulus onset) for each trial.

Trials in which electromyographic activity increased during the time window beginning 25 ms prior to the conditioned

stimulus onset through 75 ms post-onset were excluded from analysis. These trials were excluded because blinks during this interval are not considered learning-related, and can interfere with the emission of a true conditioned response eyeblink.

Conditioned responses were recorded when an eyeblink occurred between 100 and 350 ms after the tone's onset, the time interval corresponding to the 250 ms prior to the unconditioned stimulus onset. The onset latency was calculated as the time when the electromyographic activity exceeded 0.5 SDs from baseline activity.

Statistical Analysis

Block-by-block percentages of conditioned responses from dEBC experiments were fitted to growth curve models using HLM. Conditioned response averages for each of the 10 blocks for each individual were calculated and the best-fitting line was generated, resulting in one line for each participant – a total of 154 lines. Eleven from this initial group (six participants with schizophrenia and five controls) were dropped from the analysis because they failed to exhibit conditioned responding such that the difference between the last and the first estimation of a linear curve fit was <0%. Therefore, 143 participants remained for age-matching (60 in the schizophrenia group; 83 in the control group). The final sample included 59 participants with schizophrenia who were age-matched to a healthy control whose age was within 2 years of their own.

The lme function of the nlme package (53) in R 3.0 (R Development Core Team, 2009) was used to model associative learning for growth curve modeling in HLM. Models used maximum likelihood estimation, except when testing whether effects should be fixed or random, in which case restricted maximum likelihood was used as suggested by Singer and Willett (54). Linear and non-linear forms of change were examined with nested model comparisons using the likelihood ratio test. Model fit was examined with pseudo-R^2 (54), which was calculated by the squared correlation between the model's fitted and observed values, representing the proportion of variance in the outcome explained by the model.

A three-parameter logistic growth curve with a randomly varying asymptote and fixed values for the slope and inflection point was used, which fit the data well (pseudo-$R^2 = 0.73$). The model allowed different asymptote estimates across participants but not different estimates of slope or inflection point (but were allowed to differ by group). A random effect of asymptote was a better model fit than a model with a random effect of inflection point, and models with a random effect of slope did not converge. For each individual, logistic growth curves were fit to associative learning curves across the 10 blocks of the experiment. These logistic curves estimated whether the groups were different for each of the three parameters: slope, inflection point, and asymptote. The inflection point is the point on the curve where it changes curvature, and the asymptote is where learning begins to level off. The slope measures the change in associative learning over time and was used to assess differences in learning rate between groups.

We attempted to analyze data from conditioned response onset latency, but the data fit a logistic growth curve model poorly (pseudo-$R^2 = 0.24$). Therefore, although all indications were that no differences on primary dependent variables could be observed, given the lack of fit and consequent unreliability of statistical measures, we have not included this analysis in the Section "Results."

Using three separate statistical tests of between-group differences (schizophrenia vs. controls for asymptote, slope, and inflection point), a Bonferroni-corrected alpha level of $P < 0.017$ ($P < 0.05/3$ comparisons) was deemed significant, although results with $P < 0.05$ are reported.

RESULTS

Baseline Unconditioned Response Amplitude

Differences in conditioned response measurements could arise from impairment in general eyeblink performance. Therefore, to ensure that any observed differences between groups on the percentage of conditioned responses was not due to such a general performance issue, eight unconditioned stimulus air puffs were presented alone at the beginning of the experiment. Baseline unconditioned response amplitude was available for a total of 41 participants with schizophrenia and 42 controls. Neither the average peak unconditioned response amplitudes [$F(1,81) = 3.17$, $P = 0.08$] nor latencies [$F(1, 81) = 0.003$, $P = 0.96$] were significantly different between groups. While the differences in amplitude did not reach significance, it is important to note that average group differences indicated that the schizophrenia group had *larger* unconditioned response amplitudes ($M = 97.89$ μV, SD = 23.27) compared to controls ($M = 89.64$ μV, SD = 18.79). This finding is consistent with earlier findings that unconditioned response amplitude was larger on paired dEBC trials in schizophrenia (40). Overall, these findings suggest that differences in conditioned responses are unlikely to be due to deficits in blink performance in the schizophrenia group.

Percentage of Conditioned Responses

Parameter estimates of the logistic model examining learning curves of the percentage of conditioned responses are in **Table 2**. **Figure 1** shows the line fits for each participant, the group average fitted line, and the conditioned response average for each of the 10 blocks. Findings suggest that the difference in learning between the beginning and end of the experiment is similar between groups,

TABLE 2 | Parameter estimates for the HLM growth curve model for percentage of conditioned responses.

	Value (SE)	DF	*t*-value	*p*-value
$R^2 = 0.73$				
Asymptote	68.92 (3.46)	1003	−19.91	0.000
SZ-HC	−20.31 (4.97)	1003	−4.09	0.000*
Inflection Point	0.64 (0.17)	1003	3.59	0.000
SZ-HC	0.74 (0.27)	1003	2.77	0.006*
Slope	1.1 (0.19)	1003	5.88	0.000
SZ-HC	0.51 (0.33)	1003	1.59	0.112

SZ, schizophrenia, HC, healthy controls.

**Indicates differences between groups with a significance at P < 0.017.*

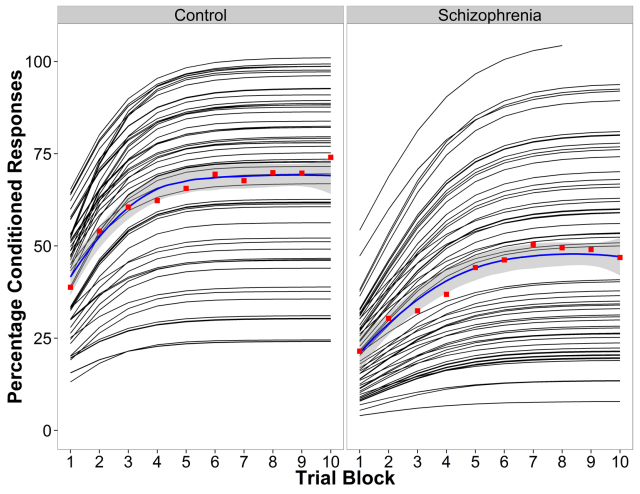

FIGURE 1 | Conditioned response data for the control group (left) and the schizophrenia group (right). The logistic curve fit for each individual (black lines), the average percentage of CRs for the raw data for each block in red, and the group average logistic curve fit in blue.

but that learning saturates later in the schizophrenia group, and the level at which saturation occurs is lower in the schizophrenia group. When the groups were considered together, performance improved across the 10 blocks of the experiment, $t(1003) = 5.88$, $P < 0.001$, SE = 0.19, and the rate of learning did not differ between groups, $t(1003) = 1.59$, $P = 0.11$, SE = 0.33. However, the asymptote was significantly lower in the schizophrenia group, $t(1003) = -4.09$, $P < 0.001$, SE = 4.97. Moreover, the inflection point occurred later in schizophrenia group [$t(1003) = 2.77$, $P = 0.006$, SE = 0.27]. These results indicate that the rate of learning over the course of the experiment (the slope), measured as the difference between blocks 1 and 10 on the fitted logistic curves, was not significantly different between groups. However, the reduced asymptote in schizophrenia makes the slope more similar between groups even though the inflection point occurred later. Overall, the schizophrenia group attained a lower ultimate level of learning and took longer to achieve this maximum.

Correlations with Clinical Symptoms

We examined associations of participants' estimates on each of the three logistic model parameters for the percentage of conditioned responses with PANSS positive, negative, general, and total scores using bivariate correlations with age partialed out. There were no significant correlations between any behavioral parameters and clinical variables.

DISCUSSION

The goal of the present study was to extend and clarify results of earlier studies examining dEBC in schizophrenia using more sophisticated statistical models. HLM of data fitted to a logistic growth curve model provided insight into how three components of the learning curve change over time in schizophrenia. Overall, associative learning in the schizophrenia group leveled off at a lower level compared to controls, and took longer to reach the maximal learning level. Surprisingly, the rate of learning (i.e., slope) within subjects with schizophrenia was not significantly different from controls.

Analysis of dEBC data using HLM, a superior analytic approach compared to ANOVA, suggests robust differences between subjects in the control and schizophrenia groups. Cerebellar abnormalities in schizophrenia are most likely responsible for these behavioral dEBC differences. The regions of cerebellar cortex that show reduced regional cerebral blood flow (rCBF) during dEBC in unmedicated schizophrenia (55) also overlap with those identified as fundamental to normal expression of conditioned eyeblink responses in animal studies (56–59). The interpositus nucleus is necessary for the acquisition and retention of the conditioned eyeblink response with cerebellar cortical sites, in particular long-term depression at the parallel fiber–Purkinje cell synapse, modulating important aspects of the gain and timing of the response (see, Ref. (60) for extensive review). Human studies of populations with cerebellar lesions or degeneration largely support these findings, and also suggest that purely cortical lesions produce significant reductions in the expression of conditioned responses, but do not abolish them (61). Importantly, cerebellar cortical structure is associated with conditioned response timing (62) and acquisition (63). Taken together, these findings suggest that abnormalities in the interpositus nuclei and the cortex of the cerebellum contribute to the dEBC deficits observed in schizophrenia.

Our laboratory has undertaken a program of research that aims to tackle outstanding questions about cerebellar abnormalities in schizophrenia. We have previously reported deficits in schizophrenia on timing tasks that rely heavily on cerebellar-based timing mechanisms, including paced finger-tapping (64) and a temporal bisection task (45, 65, 66). Using neuroimaging techniques, we can more definitively understand the extent to which the dEBC deficits in schizophrenia are uniquely attributable to alterations in cerebellar function compared to other cortical and subcortical circuits in which the cerebellum participates. We are currently using functional magnetic resonance imaging in conjunction with dEBC and paced finger-tapping to determine how cerebellar functional and structural abnormalities contribute to performance deficits in schizophrenia. Moreover, our recent studies have identified dEBC abnormalities in an intermediate phenotype of schizophrenia, namely schizotypal personality disorder (39), and in first-degree relatives of individuals with schizophrenia (44), suggesting that dEBC impairments may be risk markers for schizophrenia. Ongoing studies of first-degree relatives will determine whether familial risk is associated with morphological and functional alterations in the cerebellum and related circuits.

Our current studies and others addressing similar questions may provide evidence that the cerebellum is a potential therapeutic target for remediating symptoms of schizophrenia. Indeed, preliminary evidence supports this idea. For example, secretin is a neuropeptide with receptors in the cerebellum, which permitted us to make predictions based on a mechanistic model of its actions within the cerebellar cortex (67, 68). When we administered secretin to a small group of participants with schizophrenia, it significantly improved dEBC performance and validated the utility of the cerebellum as a potential pharmacological target

(37) [c.f., Ref. (69, 70)]. Similarly, a small sample of individuals with treatment-resistant schizophrenia underwent theta-burst transcranial magnetic stimulation of the cerebellum and experienced both improved mood symptoms and enhanced cognitive performance (71). Taken together, efforts to identify cerebellar-dependent biomarkers will facilitate the development of new potential therapeutic targets within the cerebellum that could provide previously unexplored avenues of treatment that are sorely needed for this perplexing disorder.

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Eyeblink classical conditioning and post-traumatic stress disorder – a model systems approach

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Not everyone exposed to trauma suffers flashbacks, bad dreams, numbing, fear, anxiety, sleeplessness, hyper-vigilance, hyperarousal, or an inability to cope, but those who do may suffer from post-traumatic stress disorder (PTSD). PTSD is a major physical and mental health problem for military personnel and civilians exposed to trauma. There is still debate about the incidence and prevalence of PTSD especially among the military, but for those who are diagnosed, behavioral therapy and drug treatment strategies have proven to be less than effective. A number of these treatment strategies are based on rodent fear conditioning research and are capable of treating only some of the symptoms because the extinction of fear does not deal with the various forms of hyper-vigilance and hyperarousal experienced by people with PTSD. To help address this problem, we have developed a preclinical eyeblink classical conditioning model of PTSD in which conditioning and hyperarousal can both be extinguished. We review this model and discuss findings showing that unpaired stimulus presentations can be effective in reducing levels of conditioning and hyperarousal even when unconditioned stimulus intensity is reduced to the point where it is barely capable of eliciting a response. These procedures have direct implications for the treatment of PTSD and could be implemented in a virtual reality environment.

Keywords: conditioning-specific reflex modification, explicitly unpaired, extinction, reflex modification, rabbit nictitating membrane response, virtual reality

INTRODUCTION

People exposed to trauma who suffer flashbacks, bad dreams, numbing, fear, anxiety, sleeplessness, hyper-vigilance, hyperarousal, or an inability to cope comprise the 15–25% who suffer from post-traumatic stress disorder (PTSD) (1–3). There is a crucial need to know how responding to stressful events changes as a function of trauma for patients who suffer from PTSD and particularly combat-related PTSD – a condition that can be resistant to behavioral and drug therapy (2, 4, 5). PTSD is the most common psychiatric condition for which veterans seek services (6, 7). PTSD among veterans may be 3 times higher than in the general population, although it may be 30 times higher in combat veterans (8). Even these numbers may be underestimates due to underreporting of mental disorders in active duty personnel because of perceived weakness, loss of confidence, stigma, and threat to career posed by a need for mental health services (6, 9–11). Adding further concern are recent findings that PTSD can lead to an increased risk of dementia (12, 13) and PTSD symptoms can last more than 15 years (14). Despite some progress in diagnosing and treating PTSD in civilians, treating veterans is less successful (5, 15, 16), and PTSD among veterans results in increased death (17, 18) including suicide (18, 19). It is clear every effort, including better animal modeling, needs to be made to improve our understanding and treatment of PTSD.

Researchers have developed a range of animal models of PTSD (3, 20–29). Although animal models cannot capture all the aspects of a human disorder, they are invaluable for developing and testing potential treatments, especially when a model expresses more than one phenotype of PTSD (30–33). However, many of the current animal models of PTSD have limitations. First, they focus on the fear associated with trauma (fear conditioning) without assessing or treating the hyperarousal caused by trauma or they focus on stress-induced hyperarousal without assessing or treating fear conditioning. Second, the majority of animal models rely on group data, and it is clear that not everyone exposed to trauma develops PTSD (2, 13, 30, 34, 35). In fact, depending on the population and on the type of trauma, only 5–25% of exposed people develop PTSD (1–3).

We have developed an animal model of PTSD in which conditioning and hyperarousal can both be extinguished (36). The model is based on observations that the eyeblink response becomes exaggerated as a function of classical conditioning (37–43). The exaggerated response occurs when the eliciting stimulus such as an air puff or periorbital electrical stimulation is tested by itself, and this form of hyperarousal is termed conditioning-specific reflex modification (CRM). CRM is detected by comparing responses to a range of unconditioned stimulus (US) intensities by themselves before and after classical conditioning. This phenomenon has been observed by others in rabbit eyeblink conditioning (44, 45) and in rat eyeblink conditioning (46). We now have strong evidence we can "treat" CRM as well as extinguish conditioned responses (CRs) to stimuli associated with the US. Importantly, high levels of CRM only occur in 15–25% of rabbits exposed to eyeblink classical conditioning

(EBCC) – levels that are consistent with the incidence of PTSD (2, 3, 35).

EYEBLINK CLASSICAL CONDITIONING

EBCC IN HUMANS

The history of human EBCC dates back to German studies beginning in 1899 and described by Woodruff-Pak and Steinmetz (47) who referenced an exhaustive bibliography of over 500 human EBCC studies from 1899 to 1985 compiled by Gormezano (48). EBCC in the United States was pioneered by Cason in 1922 using electric shock as the US (49). EBCC was then expanded upon by Hilgard in a subsequent series of studies in the 1930s with rats, dogs, monkeys, and humans which were all conducted with what has become the standard US for EBCC particularly in humans – a puff of air to the eye (50). The first documented studies of EBCC to investigate psychiatric disorders were published in the 1950s by Spence and Taylor when EBCC was assessed in subjects with anxiety (51) and those with neurosis and psychosis (52, 53).

The first report of EBCC in patients with PTSD was a study by Ayers and colleagues using delay conditioning in veterans (54). A number of other studies followed mostly in veterans (55–58) and one in civilians (59). The consensus of these studies is that there may be changes in EBCC as a result of PTSD but the effects are quite variable and may involve personality traits (57). These studies are reviewed in more detail in the accompanying articles from the Servatius laboratory.

EBCC IN ANIMALS

As noted above, the history of EBCC in animals began with studies using dogs in 1935, monkeys in 1936 (50), and rats in 1938 (60). Perhaps because of the strong focus on human eyelid conditioning in the intervening years (48), little if any attention was paid to EBCC in animals until the 1960s. A return to EBCC in animals may also have reflected the neurobiological limitations inherent in and the growing theoretical and methodological controversies surrounding human EBCC (47, 61, 62). To address these methodological issues as well as provide the behavioral basis for studying learning's neural substrates, Gormezano and colleagues developed classical conditioning of a series of related skeletal responses in the rabbit centered on the eyelid and nictitating membrane (63–66). These preparations were followed by the development of jaw movement conditioning, classical conditioning of an appetitive response (67), and heart rate conditioning, classical conditioning of an autonomic response (68, 69). In order to overcome the very limited ability to use invasive techniques in humans and pursue the growing interest in the neural substrates of learning, Thompson and colleagues began to use neural recording and lesion techniques to delineate the pathways and substrates of EBCC in the rabbit (70–72).

REFLEX MODIFICATION

Although the focus of nearly all classical conditioning experiments has been on the development of a CR (e.g., eyeblink) to the conditioned stimulus (CS, e.g., tone), some attention has also been paid to the unconditioned response (UR, e.g., eyeblink) to the US. For example, there is ample evidence that URs may be modified as a result of non-associative processes. Illustrated in the top panel of **Figure 1** is an example of a non-associative change in the eyeblink where repeated elicitation of the eyeblink indexed by measuring the nictitating membrane response (NMR) can lead to a reduction in the amplitude of the response known as habituation (73–81). In this example, a rabbit's response to a strong periorbital electrical stimulus (2 mA, 100 ms) decreases across four 20-trial blocks of electrical stimulation presented at different intensities (0.1, 0.25, 0.5, 1.0, and 2.0 mA) and durations (10, 25, 50, 100 ms). URs may

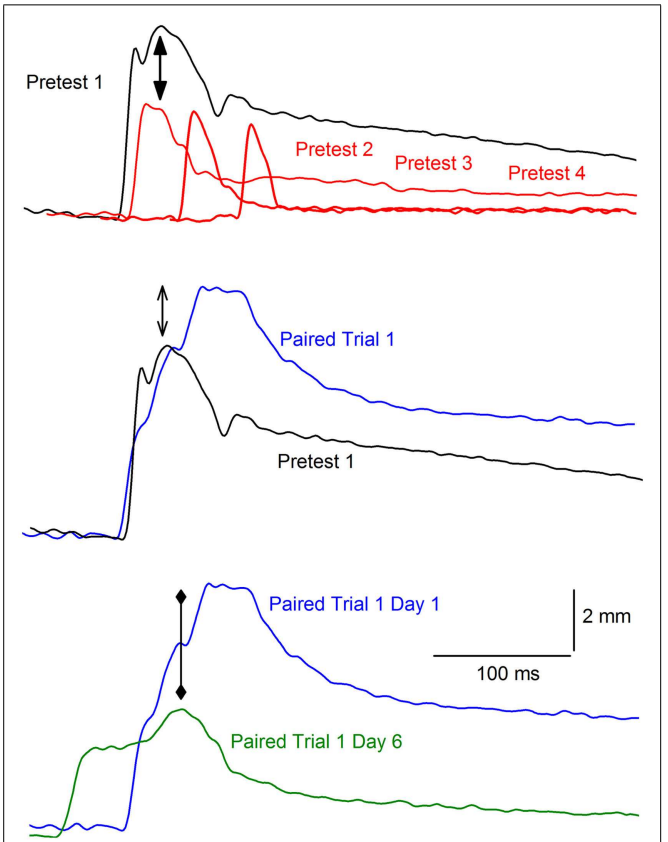

FIGURE 1 | Example of habituation and reflex modification. The top panel of the figure shows representative nictitating membrane responses (eyeblink) to a 2.0-mA, 100 ms periorbital electrical stimulus for an individual rabbit during the first (black, Pretest 1), second (red, Pretest 2), third (Pretest 3), and fourth (Pretest 4) block of pretesting to periorbital electrical stimuli of different intensities (0.1, 0.25, 0.5, 1.0, and 2.0 mA) and durations (10, 25, 50, 100 ms). The onset of the responses are staggered from left to right to help illustrate the decrease in response amplitude (solid arrows) known as habituation as a function of repeated stimulus presentations across the four blocks. The middle panel shows the response on Pretest 1 (black) compared to the response to the same 2.0-mA, 100 ms periorbital electrical stimulus on the first paired trial (blue, Paired Trial 1) of the tone conditioned stimulus and the periorbital electrical unconditioned stimulus. The open arrows indicate the increase in the amplitude of the response known as reflex facilitation on the paired trial. The bottom panel depicts the response on the first paired trial of the tone conditioned response and the periorbital electrical unconditioned stimulus on the first day (blue, Paired Trial 1 Day 1) compared to the first paired trial on the sixth and last day (green, Paired Trial 1 Day 6). The diamond arrowheads indicate the decrease in the amplitude of the response on the later paired trial when a conditioned response is present (earlier response onset). This decrease in amplitude is known as conditioned diminution.

also be enhanced or undergo sensitization; that is, a response to a weak stimulus will become larger if it is elicited after a series of stronger stimulations (82). Although non-associative, sensitization can also occur during pairings of the CS and US and can be estimated on the basis of unpaired presentations of these two stimuli (83). A CS may facilitate the rabbit NMR the first time the tone and air puff (or periorbital electrical stimulation) are presented together (that is, before any association could have formed between the two stimuli). Depicted in the middle panel of **Figure 1** is an example of an eyeblink that increased in size in the presence of a tone CS – a phenomenon known as reflex modification, in this case reflex facilitation (84–96).

Unconditioned responses may also be modified as a result of associative processes and there is substantial evidence that a UR can be modified as a function of CS–US pairings. For example, the presence of a CS may decrease the size of the UR after repeated pairings have resulted in the formation of an association. This is a phenomenon known as conditioned diminution (85, 89). The bottom panel of **Figure 1** shows an example of conditioned diminution where there is a decrease in the amplitude of the eyeblink UR from the first paired trial where there is no CR to a later paired trial where there is a CR (indicated by the earlier onset latency compared to the first trial on which only a UR is present).

In all of these aforementioned studies, the focus has been on changes in the UR that are attributable to the CS. Consequently, dependent variable measures, such as amplitude of the response, have been assessed in the presence of the CS as in the case of the bottom panel of **Figure 1**. Our original studies were influenced by the hypothesis that classical conditioning alters not only CS processing but also alters US processing. This hypothesis is consistent with a local interaction model of learning and memory in which CS and US inputs interact at a number of local dendritic sites distributed across a neuronal array (97, 98). It is from this background that we first observed the changes in the UR that has come to be termed CRM (37). By way of contrast to earlier studies where the UR was assessed in the presence of the CS, the experiments reviewed here focus on the effects of conditioning on responding to the US in the absence of the CS and, hence, examined conditioning-specific effects that are intrinsic to US processing and UR production.

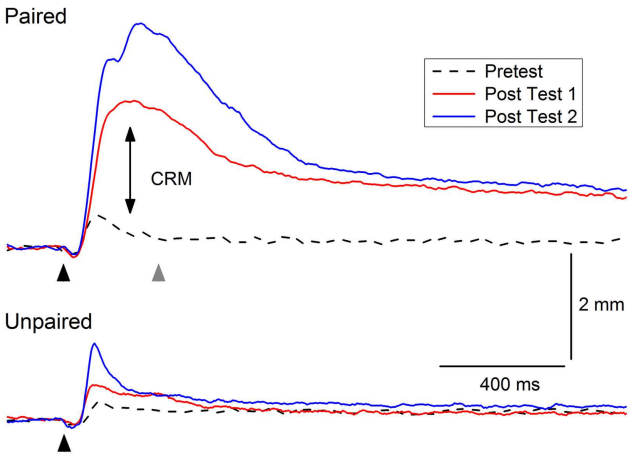

FIGURE 2 | Example of conditioning-specific reflex modification (CRM). Representative nictitating membrane responses (eyeblink) to 0.5-mA periorbital electrical stimulation (black arrowhead) averaged over four durations (10, 25, 50, 100 ms) in an individual rabbit before (dashed line, Pretest), 1 day after (red line, Post Test 1), and 1 month after (blue line, Post Test 2) 6 days of conditioned stimulus–unconditioned stimulus pairings (eyeblink classical conditioning, Paired). The responses show clear increases in amplitude, area, and peak latency (double arrow, CRM) compared to the responses of a control rabbit to 0.5-mA periorbital electrical stimulation (black arrowhead) averaged over four durations (10, 25, 50, 100 ms) before (dashed lined, Pretest), 1 day after (red line Post Test 1), and 1 month after (blue line, Post Test 2) 6 days of explicitly unpaired presentations of the conditioned stimulus and unconditioned stimulus (Unpaired). The gray arrowhead indicates where a 2.0-mA shock would have occurred during conditioned stimulus–unconditioned stimulus pairings. Although there is a slight increase in the amplitude of the response in the rabbit in the Unpaired group 1 month after explicitly unpaired presentations of the conditioned stimulus and unconditioned stimulus, it is not as large as the response seen in the rabbit from the Paired group nor is there a shift to the right in the peak latency.

CONDITIONING-SPECIFIC REFLEX MODIFICATION

THE BASIC PHENOMENON

Figure 2 shows an example of CRM in which representative NMRs to a 0.5-mA periorbital electrical stimulus are shown in a rabbit before (Pretest), 1 day after (Post Test 1), and 1 month (Post Test 2) after 6 days of EBCC (Paired). The responses show clear increases in amplitude, area, and peak latency compared to the responses in a control rabbit after 6 days of explicitly unpaired presentations of the tone CS and periorbital electrical stimulation US (Unpaired). Thus, CRM occurs following EBCC and persists for a month but does not occur following explicitly unpaired stimulus presentations – the optimal control condition for assessing non-associative contributors to responding (83). CRM is detected by comparing responses to a range of US intensities presented by themselves before and after classical conditioning and has been observed by others following EBCC in rabbits (44, 45) and rats (46). CRM is not idiosyncratic to EBCC because we have also found CRM of heart rate as a result of heart rate classical conditioning (42, 99, 100). Thus, the effect appears to exist in at least two species and in both the autonomic and the skeletal response systems. Given the subject of the present focus topic, this review will be limited to changes in the rabbit unconditioned NMR that occur as the result of EBCC because CRM of HR is obtained at conditioning parameters (i.e., long interstimulus intervals) that do not normally support EBCC. The NMR serves as a convenient index of the eyeblink as it is a component of the defensive response system consisting of closure of the upper eyelid, retraction of the eyeball, and sweep of the nictitating membrane which are very highly correlated (63, 65, 101).

BEHAVIORAL LAWS

Rabbit EBCC has yielded a large number of behavioral "laws" that have been enumerated and detailed elsewhere (63, 66, 69, 102, 103). Chief among these "laws" is the relationship between the strength and rate of EBCC and a number of parameters including CS and US intensity and duration, interstimulus interval, and number of stimulus pairings (66). In a series of experiments reviewed previously (40, 42), we have found that CRM is also a function of a

number of parameters including the nature (air puff and periorbital electrical stimulation) and intensity of the US (39, 104), the interstimulus interval (105), and the number of pairings (37, 38).

STIMULUS GENERALIZATION

Another important phenomenon in rabbit EBCC that has been observed in other species and behavioral paradigms is generalization – responding to stimuli similar to the stimulus used during EBCC (106–108). CRM by its very nature is generalization along the intensity dimension of the US for both electrical stimulation and air puff (39). Due to a ceiling effect for the highest intensities of periorbital electrical stimulation, the strongest levels of CRM are detected below the training intensity (37–39). This is not the case for the weaker stimulation afforded by air puff where CRM occurs at high as well as moderate stimulus intensities (39). We have found that CRM can generalize from periorbital electrical stimulation to air puff but does not generalize from air puff to periorbital shock which seems to reflect the need for an intense US to support CRM (39) making it relevant for modeling PTSD.

CONTEXT

Previous experiments suggest that CRM obeys behavioral laws similar to those of classical conditioning and, like classical conditioning, CRM is sensitive to a shift in context (41). In a series of experiments the auditory, olfactory, tactile, and visual properties of the context in which rabbits were given EBCC and CRM testing were manipulated to determine the effects of context on the level of CRM. An initial experiment demonstrated that when CRM was tested in a novel context, CRM levels were as strong as when testing occurred in the familiar, EBCC training context. To factor out differences in the amount of exposure to the different contexts that may have explained the results of the first experiment, exposure to all contexts was equated in a second experiment. The results showed that there was less CRM when testing took place in a context that was equally familiar but different from the EBCC training context. A context-dependent reduction in responding during EBCC has been demonstrated in rabbits that showed a drop in conditioned responding of 50% when given pairings in a different context where the visual, tactile, and olfactory characteristics had been altered from the original training context (109). The reduction in responding as a result of a context shift during rabbit EBCC has been reported in other learning paradigms including fear conditioning (110, 111), taste aversion learning (112), and conditioned suppression (113). Consistent with this context shift effect, our context experiments show that if exposure to the contexts is equated (111), CRM can be significantly reduced, but not eliminated, by a shift in the context from training to testing.

RESILIENCE AND SUSCEPTIBILITY

Examination of individual subject data across CRM studies revealed CRM is not an all-or-none phenomenon with considerable between-subject variability in the presence and degree of CRM. Although some CRM occurs in over 50% of rabbits, high levels of CRM (one standard deviation above mean percent change) only occur in 15–25% of rabbits even though all reach conditioning levels in excess of 85% CRs. **Figure 3** shows an example of the extremes in responding by two different rabbits to the same 0.5-mA periorbital electrical stimulus. Despite high, almost identical levels of EBCC (100 vs. 98.5% CRs), these two subjects show profound differences in their responses to the periorbital electrical stimulus on Post Test. The first subject shows particularly strong CRM and would be considered "susceptible" whereas the second subject shows no CRM at all and would be considered "resilient." In 135 subjects trained with our standard EBCC paradigm consisting of 80 daily presentations of a 400-ms, 82-dB, 1,000 Hz tone CS that coterminates with a 100-ms, 2.0-mA, 60-Hz periorbital electrical stimulus, we found the strongest predictor of CRM (indexed by an increase in response magnitude and area) was short CR onset latency (43). We also found that during periorbital electrical stimulation on Pretest, the strongest predictor of subsequent CRM was response onset and peak latency – the faster the rabbit's response, the more likely it was to develop CRM. Therefore, the speed with which a rabbit responds to the CS during training and to the periorbital electrical stimulus during pretest are good predictors of CRM and are indices of susceptibility. This would correspond to differences in reaction time in PTSD – something that is not often observed (114–116) but has been reported (117).

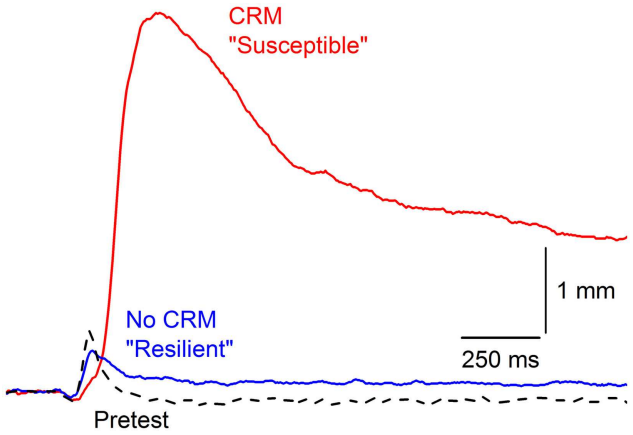

FIGURE 3 | Susceptibility and resilience of conditioning-specific reflex modification. Representative nictitating membrane responses (eyeblink) to 0.5-mA periorbital electrical stimulation averaged over four durations (10, 25, 50, 100 ms) in two individual rabbits that show very different responses to the same 0.5-mA US on Post Test after having been given 6 days of conditioned stimulus–unconditioned stimulus pairings (eyeblink classical conditioning) at the same time to essentially the same high levels (100 vs. 98.5% conditioned responses). The rabbit with the larger, later response (red line) is considered "Susceptible" with a 2000% increase in response amplitude whereas the rabbit with a response that did not differ from Pretest is designated "Resilient" (dashed line). Figure adapted from Smith-Bell et al. (43), Copyright 2012 by the American Psychological Association.

INCUBATION

The symptoms of PTSD do not always occur immediately after trauma and can become more pronounced over time. A delay in the onset of symptoms by as much as 6 months has been incorporated into previous diagnostic criteria of PTSD (118, 119), but there is now debate about whether delayed-onset PTSD actually exists in either veterans or civilians with evidence for both points of

view (118–124). In our animal model of PTSD symptoms, rabbits do not show a delay in onset of CRM, but there is a window during which incubation exacerbates CRM. The results are consistent with clinical data in which exacerbation or reactivation of prior symptoms accounts for 38.3% of military cases of PTSD and 15.3% of civilian cases (120, 125). In one set of experiments, we have observed the exacerbation of symptoms as a function of a period of incubation (126). CRM typically requires at least 3 days of EBCC when levels of conditioning reach or exceed 85% CRs (37, 39). We carried out an experiment (**Figure 4**) in which rabbits were given EBCC for just 1 day resulting in mean conditioning levels of only 45% CRs, and saw little evidence of CRM when tested the next day. However, if left in their home cages for 6 days, there was a significant amount of CRM which persisted for a week after testing (126). The incubation effect was not strong following 10 days in the home cage and did not persist. These data suggest there may be no delay in CRM onset but there is a window for incubation to exacerbate CRM.

RESPONSE GENERALIZATION

One of the most interesting aspects of our initial CRM experiments was the observation that, in individual subjects, responses to weak periorbital electrical stimulus intensities appeared to have a significantly different topography after EBCC than they do before EBCC and that the topography was reminiscent of the CR (37, 40). This observation was even more clearly articulated by Gruart and Yeo (44) when they first reported changes in the rabbit eyelid UR following EBCC. The marked alteration in response topography is somewhat lost in the averaging that takes place when presenting group data especially when, as noted above, not all rabbits show CRM. **Figure 5** shows the strong similarity between a CR that occurs during EBCC and a UR to periorbital stimulation by itself assessed after EBCC compared to an UR assessed before EBCC. These early observations lead to the hypothesis that CRM is a CR that generalized from the CS–US pairings to the US itself (40, 44). A series of experiments were conducted to test this hypothesis by altering the topography of the CR by presenting two shocks during CS pairings or by presenting CS–US pairings with two different interstimulus intervals (38). The results provided evidence both for and against the hypothesis so a final experiment was designed to eliminate CRs by presenting the CS by itself during extinction (38). If the exaggerated responses to the US after EBCC (CRM) were generalized CRs, it was reasoned that eliminating the CRs should eliminate CRM. The results of this experiment were more conclusive. Despite reducing CRs to essentially baseline levels of less than 10% by presenting the CS by itself, **Figure 6** shows CRM remained virtually intact. A number of control groups actually proved to be even more instructive. First, presentations of the US by itself completely eliminated CRM as shown in **Figure 6** but left CRs relatively intact. Thus, the extinction of CRs left strong levels of CRM and the extinction of CRM left strong levels of conditioned responding. Second, combining presentations of the CS and the US in an explicitly unpaired manner resulted in elimination of

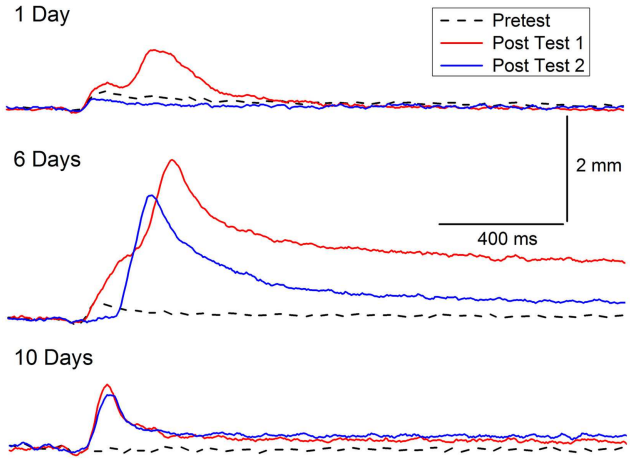

FIGURE 4 | Incubation of conditioning-specific reflex modification. Representative nictitating membrane responses (eyeblink) to a 0.25-mA periorbital electrical stimulus averaged over four durations (10, 25, 50, 100 ms) in individual rabbits before (dashed line) and after 1, 6, or 10 days of incubation in the home cage (red line) following a single session of conditioned stimulus–unconditioned stimulus pairings (eyeblink classical conditioning) which supported a level of only 45% conditioned responses. The blue lines depict nictitating membrane responses to 0.25-mA periorbital electrical stimulation 7 days (of incubation) after Post Test 1. Although there is some suggestion of conditioning-specific reflex modification after 1 and 10 days of incubation, there was very clear and strong conditioning-specific reflex modification that occurred after 6 days of incubation and persisted a week later. Figure adapted from Schreurs et al. (126), used with permission from Elsevier.

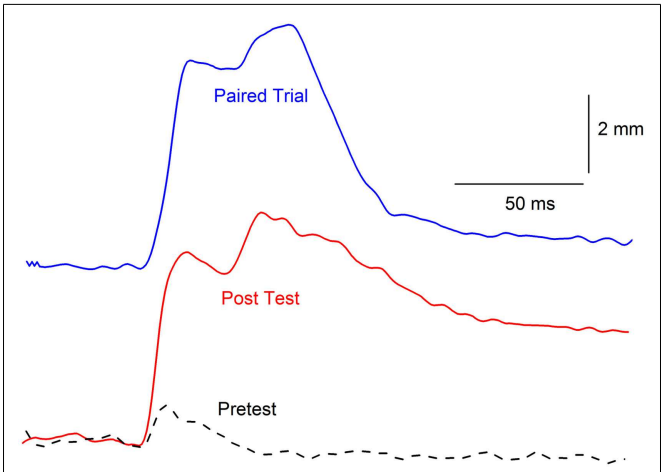

FIGURE 5 | Topographical similarity between a conditioned response and conditioning-specific reflex modification. Representative nictitating membrane responses (eyeblinks) in the same rabbit to a tone paired with shock during the third day of conditioned stimulus–unconditioned stimulus pairings (eyeblink classical conditioning, blue line, Paired Trial) and 0.5-mA periorbital electrical stimulation presented by itself before (dashed line, Pretest), and after (red line, Post Test) 6 days of conditioned stimulus–unconditioned stimulus pairings. The response after eyeblink classical conditioning shows a strong similarity in response amplitude, peak latency, and overall topography compared to the response before eyeblink classical conditioning. The responses are shifted in time so that their onsets coincide even though the response on the paired trial is to the conditioned stimulus that overlaps with the periorbital electrical stimulus and the responses on the Pretest and Post Test trial are to 0.5-mA periorbital electrical stimulation by itself.

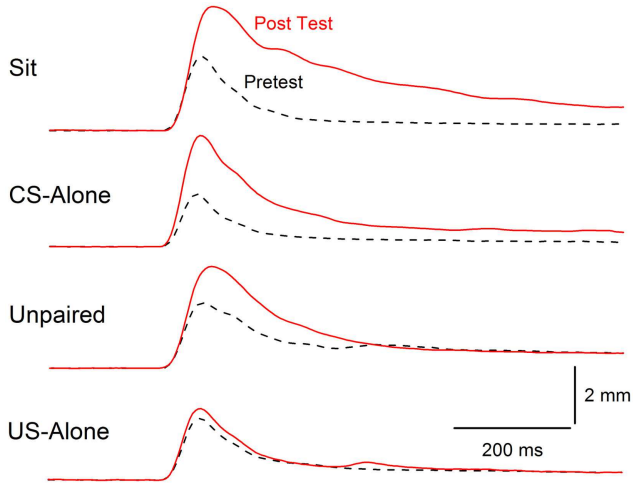

FIGURE 6 | Extinction of conditioning-specific reflex modification. Averaged nictitating membrane responses (eyeblink) to a periorbital electrical stimulus of 1.0 mA averaged over four durations (10, 25, 50, 100 ms) for four groups of rabbits before 6 days of conditioned stimulus–unconditioned stimulus pairings (eyeblink classical conditioning) (dashed lines, Pretest) and 6 days after (red line, Post Test) either exposure to the training chamber with no further stimulus presentations (Sit), presentations of the conditioned stimulus alone (CS-alone), explicitly unpaired presentations of the conditioned stimulus and unconditioned stimulus (Unpaired), or presentations of the unconditioned stimulus alone (US-alone). The level of conditioning-specific reflex modification (CRM) was highest in the Sit group, followed by the CS-alone group, and the Unpaired group with virtually no CRM in the US-alone group. Although there was residual CRM in the Unpaired group, rabbits in this group showed no responding to the conditioned stimulus unlike rabbits in the Paired and US-alone groups suggesting that only unpaired presentations of the conditioned stimulus and unconditioned stimulus were able to significantly reduce CRM as well as eliminate conditioned responses to the conditioned stimulus. Figure adapted from Schreurs et al. (38), in the public domain.

CRs and a reduction in the level of CRM (**Figure 6**). It was these experiments that led to a further exploration of treatments that eliminate both CRs and CRM as a possible treatment strategy for PTSD.

EXTINCTION OF CRM

There is a significant body of evidence from both clinical and basic research that repeated presentation of feared stimuli does not prevent fear from returning – a phenomenon referred to as "relapse" (127, 128). Nevertheless, fear extinction is a cornerstone of many approaches to the treatment of PTSD (3, 28, 129–137). However, the renewal of fear or relapse may be "thwarted" by unpaired presentations of both the feared stimulus and the event producing the fear (38, 138–140). Experiments drawn from a large number of different conditioning paradigms including human and rabbit EBCC (36, 39, 141–144), as well as conditioned bar-press suppression in rats (138, 139), and human discriminative fear conditioning (140) show unpaired presentations of the CS and US produce extinction of a CR. In the human discriminative fear study, Vervleit and coworkers found that compared to normal extinction, only unpaired extinction prevented renewal of fear responses in people trained to discriminate one of two pictures paired with shock (140).

In rabbit experiments designed to extinguish EBCC, comparable extinction of responding to the CS occurs following CS-alone or unpaired CS and US presentations (38). However, as noted above and shown in **Figure 6**, unpaired presentations were able to extinguish CRM better than CS-alone presentations (38). The ability of unpaired presentations to diminish both CRs and exaggerated URs (i.e., CRM) suggests it may be relevant for treating both the conditioned fear and hyperarousal symptoms of PTSD (41, 42, 104). However, no matter how effective unpaired extinction might be in extinguishing fear and hyperarousal in animal models, it would be ethically unacceptable for treating PTSD because the US intensity used in unpaired extinction has always been the same as that used to induce classical conditioning (36, 39, 138–144). The repeated presentation of a traumatic event responsible for PTSD in order to treat it is untenable.

UNPAIRED EXTINCTION THAT IS CLINICALLY RELEVANT

To address concerns about using a traumatic stimulus during unpaired extinction and make an unpaired extinction procedure more clinically relevant, rabbit EBCC experiments were conducted in which unpaired extinction sessions employed periorbital electrical stimulation of reduced intensity that was presented for different numbers of days (36). Specifically, rabbits received US testing (Pretest), EBCC, another session of US testing to determine the size of CRM (Post Test 1), and then 1, 3, or 6 days of unpaired CS and US presentations with a weak (0.25 mA), moderate (1.0 mA), or strong (2.0 mA) US followed by a final session of US testing to determine the effect of unpaired presentations on CRM (Post Test 2). The results revealed extinction of both CRs and CRM was a function of the US intensity used during unpaired stimulus presentations and the number of days of those unpaired stimulus presentations (36). The levels of CRs declined from 95% to less than 20% within 3 days of unpaired stimulus presentations. **Figure 7** shows CRs during acquisition and 1, 3, or 6 days of unpaired extinction in which the US intensity was eight times weaker (0.25 mA) than the intensity used during pairings (2.0 mA). **Figure 8** depicts sample responses from different rabbits before and after EBCC (Pretest and Post Test 1, respectively) and again after unpaired stimulus presentations (Post Test 2) with a 0.25-mA US that were delivered for either one, three, or six daily sessions (days). The sample responses in the middle and right illustrate that after as few as three sessions of unpaired presentations with a weak US, any CRM seen after EBCC (red lines) was largely eliminated (blue lines). In contrast, the sample responses on the left show clearly that CRM was actually enhanced after a single session of unpaired presentations with a weak US. Taken together, these data suggest that both CRs and CRM seemed to be diminished, if not eliminated, most effectively with at least 3 days of mild US presentations but one session of stimulus presentations actually appears to exacerbate responding. Of note, and of particular clinical relevance, was the finding that extinction of CRs and CRM occurred even though the weak US produced relatively low levels of responding (rabbits blinked to the weak US on less than 25% of occasions). Analysis of rabbit heart rate during these sessions indicated that this weak US did not produce any

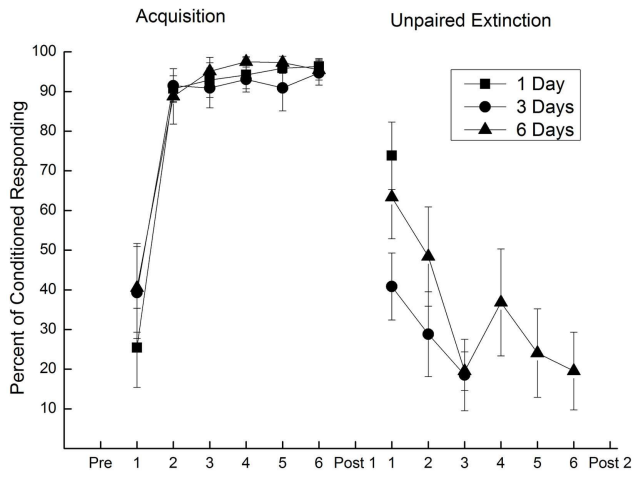

FIGURE 7 | Extinction of conditioned responding with unpaired presentations of a conditioned stimulus and weak unconditioned stimulus. Mean (±SEM) conditioned responses to the conditioned stimulus during 6 days of pairings of a conditioned stimulus and a 2.0-mA unconditioned stimulus (Acquisition) and during one (square), three (circle), or six (triangle) subsequent days of unpaired conditioned stimulus–unconditioned stimulus presentations (Unpaired Extinction) with a 0.25-mA unconditioned stimulus. Conditioned responding increased to asymptotic levels of 95% during Acquisition and fell to less than 20% following 3 days of unpaired presentations (Unpaired Extinction). Figure adapted from Schreurs et al. (36), used with permission from Elsevier.

change in heart rate, suggesting it was not unduly stressful (36). One important implication of these data is that treatment must not be brief because brief treatment using unpaired stimulus presentations may not just be ineffectual; it may actually heighten the symptoms of PTSD.

VIRTUAL REALITY

If weakened versions of the initiating trauma are to be used as part of PTSD therapy, there would be very few such events that could or even should be repeated or recreated. The advent of credible virtual reality (VR) environments that have been developed to treat PTSD provide a feasible way around this stricture (145–151). Given the unpaired extinction data reviewed above, one could imagine a treatment situation in which a PTSD patient could be asked to describe a specific trigger or set of triggers for unwanted memories (150) and present the trigger(s) in an unpaired manner with a weakened version of an aversive event. A weakened but still stressful version of an explosion might be strongly shaking a driver's seat in a virtual Humvee which is part of a VR scenario in which the sights and sounds of combat are also presented (149–151). The VR environment could be programed to present these events in a separate, unpaired manner and the prediction would be that, with a number of repetitions over more than one session, PTSD symptoms would abate. For example, the sights, sounds of a previously traumatic context could be presented, and then the goggles and headphones would go blank and silent for a period of no stimulation which would then be followed by the driver's seat being strongly shaken. The sequence of these series of events would be randomized so that they would never occur together to reflect the explicitly unpaired procedure (83). Importantly, given that CRM has been shown to generalize from stressful periorbital electrical stimulation to what would be considered less stressful air puffs, the weakened versions of stressful events used in an unpaired extinction procedure may not need to involve the traumatic event. Psychophysiological indices including heart rate, skin conductance, respiration, and cortisol levels could be used to assess stress levels and titrate the intensity of the stimulation.

METHODOLOGICAL ADDENDUM

STIMULUS DELIVERY AND RESPONSE MEASUREMENT

The experiments described in this review require precise control and calibration of stimulus parameters particularly intensity and timing of the US. This is relatively straightforward for periorbital electrical stimulation through the use of programmable shock delivery equipment and the use of digital computer control. On the other hand, the delivery of air puff requires more elaborate equipment and techniques including a digitally controlled, programmable pressure regulator and an accurate digital manometer to ensure that the intensity of the air puff reflects the air striking the cornea and not the pressure at the source. Response detection is also of importance especially if response characteristics such as latency, amplitude, and area are to be determined in addition to simply registering if a response occurs or not. As a result, transduction and recording of the eyeblink response becomes important. Researchers may wish to consider the advantages and disadvantages of remotely sensing versus directly measuring the closure of the eyelid using mechanical coupling. For example, infrared reflectance measures may not be capable of completely quantifying the peak latency of a response whereas mechanical couple may produce drag that subtly alters the latency and amplitude of a response (152). EMG recording of the *orbicularis oculi* muscle may have advantages but the electrical noise induced by periorbital electrical stimulation as well as time constants of integration affecting onset latency and difficulty in determining units of response amplitude present limitations in quantifying the UR.

DATA ANALYSIS

Even if the UR is transduced accurately, questions remain about the analysis of data, particularly when responses are at the limits of detectability as the result of very weak stimulation. By convention and due, in part, to the limits of analog instrumentation, an NMR or eyeblink response has been defined as movement of at least 0.5 mm (61, 66, 153, 154). How then is a change in response amplitude and latency from pretest to post test determined if there is no response on pretest but a significant response on post test as often occurs after EBCC? The main issue has always been what to do about the lack of a response on pretest or post test. We have addressed this in several ways including analyzing individual subject data only for US parameters at which responses occurred (37–39, 104), averaging topographies across subjects and analyzing for changes in skew and kurtosis (41, 155), and calculating percent change where a response on a test was considered to be a 100% change if there was no response on the other test (43). Most recently, two additional measures, magnitude of the response and magnitude of the response area, have been calculated to overcome the limitations of empty data cells on pretest or post test resulting

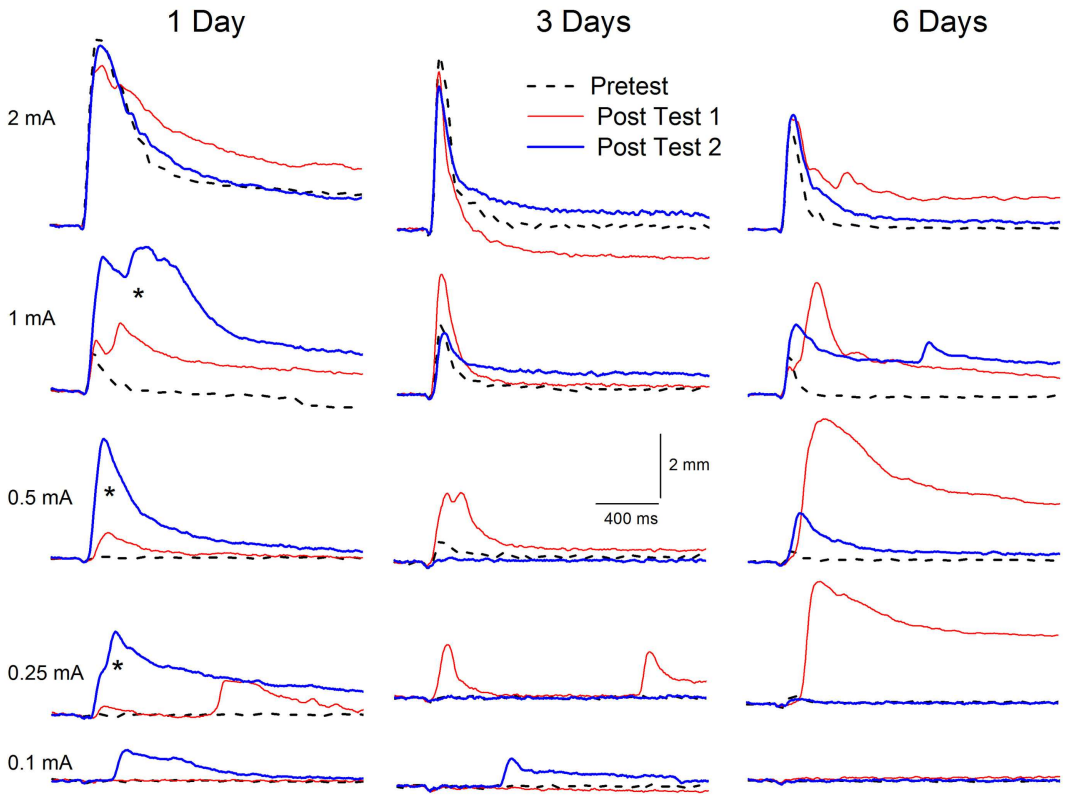

FIGURE 8 | Extinction of conditioning-specific reflex modification with a weak unconditioned stimulus. Representative nictitating membrane responses (eyeblink) to a range of periorbital electrical stimulus intensities (0.1, 0.25, 0.5, 1.0, and 2.0 mA) averaged over four durations (10, 25, 50, 100 ms) in individual rabbits before (dashed line) and 6 days after (red line) conditioned stimulus–unconditioned stimulus pairings (eyeblink classical conditioning), and again after (blue line) 1, 3, or 6 days of explicitly unpaired stimulus presentations of the conditioned stimulus and a weak unconditioned stimulus (0.25 mA) that was 87.5% weaker than the periorbital electrical stimulation previously used to establish eyeblink classical conditioning (2.0 mA). Conditioning-specific reflex modification (CRM) was established as a result of the 6 days of eyeblink classical conditioning [comparison of Pretest (dashed lines) to Post Test 1 (red lines) for intensities below the training intensity of 2.0 mA]. Following as few as three subsequent days of explicitly unpaired stimulus presentations of the conditioned stimulus and weak unconditioned stimulus, the levels of CRM (Post Test 2, blue lines) were all lower than after conditioning (Post Test 1, red lines) and in many cases returned toward baseline levels (Pretest, dashed lines). Importantly, the level of CRM increased significantly (asterisks) after just 1 day of explicitly unpaired stimulus presentations of the conditioned stimulus and weak unconditioned stimulus. Figure adapted from Schreurs et al. (36), used with permission from Elsevier.

from subthreshold URs, particularly at lower US intensities and durations (36, 43, 126). Magnitude of the response and magnitude of the response area have included the amplitudes and areas of all nictitating membrane movements above baseline and provide the most procedurally neutral estimates of responding (154).

CONDITIONED RESPONSE DEFINITION

Another issue in data analysis turns upon the practice of categorizing responses as CRs if they are "adaptively timed," a term based on the onset latency of responses (this is probably wrong anyway because one should be looking at the latency of the peak to coincide with US delivery but that would require CS-alone test trials that are un-confounded with the UR to the US which many experiments do not include). The concept of adaptively timed responses is based on the notion that CRs lessen or even avoid the aversiveness of the US when the maximum closure of the eyelid coincides with the occurrence of the US. This adaptive response may therefore be argued as being reinforcing, adding an instrumental component to CRs also known as the "law of effect" (156–158). Coleman has reviewed the literature on the "law of effect" and conducted an experiment showing quite clearly that, at least in rabbit EBCC, the imposition of a contingency between the occurrence of a CR and a reduction in the intensity of a shock US results in less rather than more responding – a finding that completely contradicts a "law of effect" prediction (156). In other experiments, including tail flexion in the rat (159), appetitive jaw movement conditioning in rabbits (160) and human EBCC (157), the lack of significant effect and even inferior conditioning of subjects explicitly designed to benefit from the "law of effect" is clear (157, 159, 160). In contrast, early experiments by Schlosberg were interpreted as "successful" only if CRs modified the US (60, 161). In fact, Schlosberg used the term "adaptive" in describing responses that had an effect on the US and "non-adaptive" for those that did not (p. 383). The pervasiveness of this assumption about the "role" of the occurrence and timing of CRs and its periodic reintroduction (162) may account for more modern EBCC experiments in which responses

are only considered to be CRs if they occur within an interval that is characterized as "adaptive."

The use of onset latencies to detect adaptively timed CRs and hence, "true CRs" can be traced to another period in the history of EBCC where latencies were used to identify and eliminate the data of "voluntary responders" (62, 163, 164). Voluntary responders were subjects who were "rejected" from experiments based on the occurrence of short-latency eyeblinks that occurred between 200 and 300 ms after CS onset and were judged to have the same appearance as subjects who were instructed to blink or by subjects who reported they were blinking "voluntarily" to avoid the air puff (165). This practice has been explicitly adopted by a number of laboratories especially during trace conditioning where there was a long interval between the offset of the CS and the onset of the US because it "corrected for both voluntary and random blinks that could occur as a result of the longer trace intervals" (166, 167).

In our view, an empirical approach to determining onset latency needs to be neutral with respect to characterizing responses. We endorse the complete characterization of all responses using a range of dependent variables including onset and peak latency and presenting all response onsets on a latency histogram without any preconceptions of how responses should look or be distributed. Publication of such histograms together with any interpretation of what are considered responses whether they be "adaptive" or not would allow readers to interpret the data for themselves.

SUMMARY AND CONCLUSION

There is a crucial need to know how responding to stressful events changes as a function of trauma for those who suffer from PTSD. A number of treatment strategies for PTSD are capable of treating only some of the symptoms because the extinction of fear does not deal with the various forms of hyper-vigilance and hyperarousal experienced by people with PTSD, especially in combat veterans. Based on our work on conditioning of the rabbit's NMR, we have developed a preclinical EBCC model of PTSD that addresses both CRs to trauma-associated cues as well as hyperarousal (CRM). Animal models of EBCC are particularly useful here because EBCC is one of the few behavioral paradigms in which there is a one-to-one correspondence between animals and humans. We have demonstrated that CRM follows many of the same behavioral rules as EBCC, can generalize across stimulus modalities, shows sensitivity to context manipulations, and can be exacerbated after an incubation period. Importantly, CRM does not develop in all animals just as PTSD does not develop in all those exposed to trauma, with some individuals demonstrating susceptibility while others show resilience. We have shown that CRs and CRM can be simultaneously extinguished by unpaired stimulus presentations, even when US intensity is reduced to the point where it is barely capable of eliciting a response. This is important because presenting strong unconditioned stimuli as a therapeutic approach would be untenable. These unpaired procedures with attenuated stimuli have direct implications for the treatment of PTSD and could be implemented in a VR environment.

AUTHOR CONTRIBUTIONS

BS and LB conceived and wrote the manuscript.

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Investigating the role of hippocampal BDNF in anxiety vulnerability using classical eyeblink conditioning

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Dysregulation of brain-derived neurotrophic factor (BDNF), behavioral inhibition temperament (BI), and small hippocampal volume have been linked to anxiety disorders. Individuals with BI show facilitated acquisition of the classically conditioned eyeblink response (CCER) as compared to non-BI individuals, and a similar pattern is seen in an animal model of BI, the Wistar-Kyoto (WKY) rat. The present study examined the role of hippocampal BDNF in the facilitated delay CCER of WKY rats. Consistent with earlier work, acquisition was facilitated in WKY rats compared to the Sprague Dawley (SD) rats. Facilitated acquisition was associated with increased BDNF, TrkB, and Arc mRNA in the dentate gyrus of SD rats, but learning-induced increases in BDNF and Arc mRNA were significantly smaller in WKY rats. To determine whether reduced hippocampal BDNF in WKY rats was a contributing factor for their facilitated CCER, BDNF or saline infusions were given bilaterally into the dentate gyrus region 1 h prior to training. BDNF infusion did not alter the acquisition of SD rats, but significantly dampened the acquisition of CCER in the WKY rats, such that acquisition was similar to SD rats. Together, these results suggest that inherent differences in the BDNF system play a critical role in the facilitated associative learning exhibited by WKY rats, and potentially individuals with BI. Facilitated associative learning may represent a vulnerability factor in the development of anxiety disorders.

Keywords: hippocampus, dentate gyrus, TrkB, Arc, Wistar-Kyoto rat

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Introduction

Anxiety is the most commonly treated and prescribed for psychiatric condition in today's society. Determining who is susceptible to developing anxiety disorders and how these vulnerabilities impact treatment efficacy is currently an active area of research. Individual differences play a crucial role in whether a person develops an anxiety disorder or not. Epidemiologic studies indicate that exposure to early childhood trauma and chronic stress increases one's risk to developing anxiety disorders, whereas a behaviorally inhibited temperament, a small hippocampal volume, and more recently, dysfunction of hippocampal brain-derived neurotrophic factor (BDNF) are associated with inherent vulnerabilities. While various risk or vulnerability factors have been identified, the mechanisms by which they confer vulnerability are still unknown (1, 2).

Brain-derived neurotrophic factor is a neurotrophin that influences cell growth, cell differentiation, and synaptic modification (3, 4) and is highly expressed in the developing and adult hippocampus (5–8). Recently, a single nucleotide polymorphism (SNP) of the coding region of the BDNF gene (Val66Met) has been identified as a risk factor for anxiety disorders, including post-traumatic stress disorder (PTSD) (9, 10). The genetic variation resulting in a substitution of a valine to a methionine at codon 66 restricts intra-cellular trafficking and activity-dependent release of hippocampal BDNF. Individuals with the met allele have reduced hippocampal volume (11–14), deficits in hippocampal-dependent memory (15, 16), and altered responses to emotional stimuli (17, 18). Given that BDNF is released in an activity-dependent manner, BDNF may be a key factor in experience-dependent vulnerability to psychiatric disorders (19).

The link between an abnormal BDNF system and anxiety vulnerability may be through the hippocampus. A small hippocampal volume and impaired hippocampal-dependent learning are likely pre-existing conditions in those that develop PTSD, suggesting that a dysfunctional hippocampus is a vulnerability factor for PTSD (2, 20). PTSD patients with the Val66Met SNP were less responsive to cognitive behavioral therapy than those without the SNP (21), implicating an involvement of BDNF in extinction learning. In humans, abnormally low levels of BDNF are associated with a smaller hippocampal volume (22) and mood disorders including obsessive-compulsive disorder (23), and depression (24). The link between low levels of hippocampal BDNF and mood disorders has been dubbed the neurotrophin hypothesis, whereby enhancement in BDNF signaling is observed in the hippocampus after administration of antidepressants (25, 26). These results suggest an association between dysfunction of the BDNF system, small hippocampal volume, hippocampal learning impairment, and risk to develop mood disorders in humans.

Similar to humans, BDNF is important for normal function of the hippocampus in animals. A low amount of BDNF is associated with a smaller hippocampal volume (22). BDNF is important for adult neurogenesis in the dentate gyrus (27), and reduced BDNF impairs spatial memory and extinction of fear memories (28). Anxiety-related behaviors are also enhanced in the transgenic mouse reproducing the Val66Met SNP (Met66 allele) of humans (9, 29). These mice have smaller hippocampi, reduced activity-dependent secretion of BDNF, dendritic shrinkage in the DG, and impaired extinction of fear conditioning compared to wild-type mice. The Val66Met polymorphism has also been linked to reductions in NMDA transmission, and resistance to selective serotonin reuptake inhibitor (SSRI)-induced LTP and neurogenesis in the dentate gyrus (30, 31). Thus, low levels of BDNF protein or impaired BDNF release via a Val66Met SNP results in a smaller hippocampus, abnormal fear extinction, anxiety-related behaviors, and reduced efficacy of antidepressants.

Behavioral inhibition is a temperament characterized by withdrawal from and avoidance of novel social and non-social interactions (32) and is a vulnerability factor for developing anxiety disorders (33–35). The neurobiology of inhibited temperament has been heavily linked to alterations in amygdala, prefrontal cortex, and basal ganglia (36). Although less well-studied with respect to inhibited temperament, the hippocampus also demonstrates altered function in individuals with inhibited temperament (36). In particular, the interaction of the risk factor of childhood maltreatment and the inherent vulnerability of inhibited temperament was associated with increased activation of the hippocampus to novel faces with the strongest correlation in individuals who developed an anxiety disorder (37). Importantly, the activity in the amygdala to novel faces did not correlate to childhood maltreatment, suggesting the amygdala and hippocampus may contribute differently to inhibited temperament.

Reflective of altered hippocampal function in behavioral inhibition is the facilitation of non-hippocampal-dependent associative learning in individuals with inhibited temperament. The delay paradigm of classical conditioning of the eyeblink response (CCER) does not require the hippocampus (38), in contrast to the trace paradigm of CCER. In fact, hippocampal damage can facilitate acquisition of delay CCER (39), whereas similar damage impairs acquisition of trace CCER (40). Support that inhibited temperament is associated with hippocampal dysfunction is the finding that individuals scoring high on behavioral inhibition scales acquire delay classical conditioning faster than non-inhibited individuals (41–44). Similarly, the Wistar-Kyoto (WKY) rat, an animal model of behavioral inhibition, demonstrated facilitated acquisition of delay CCER (45). Thus, behaviorally inhibited temperament is associated with facilitated associative learning that may underlie anxiety vulnerability (46).

The WKY rat demonstrates inhibited temperament as evidenced by reduced exploration in the open-field test (47, 48) and freezing behavior in response to novel social and non-social stress (48, 49). Additionally, WKY rats are hyper-sensitive to stress (50–52) and acquire active avoidance more rapidly, to a greater extent, and more persistently than Sprague Dawley (SD) rats (53, 54). Avoidance is a common feature of all anxiety disorders, and greater persistent avoidant responding is reminiscent of individuals with anxiety disorders (55). The WKY rat has a smaller hippocampal volume compared to the non-inhibited rat strains (56), is impaired in hippocampal-dependent learning tasks (49, 57), and behaves similarly to rats with hippocampal damage (56, 58). The BDNF system may be abnormal in the WKY rat; serum BDNF levels in WKY, but not SD, rats decreased following stress (59), and SSRIs are less effective in WKY rats compared to SD rats in the Porsolt Swim test (60), similar to mice with low levels of BDNF or Val66Met SNP.

In summary, an impaired BDNF system is a vulnerability factor for anxiety disorders and affects normal hippocampal function. Inhibited temperament is also a vulnerability factor for anxiety disorders and is associated with facilitated acquisition of delay CCER in humans and animals. The present study was conducted to determine whether an impaired hippocampal BDNF system underlies facilitated CCER that is associated with inhibited temperament and anxiety vulnerability.

Materials and Methods

Subjects were male SD and WKY rats obtained from Charles River, Kingston, NY, USA. They were approximately 3 months in age at the time of testing and maintained on a 12-h light/dark cycle with onset of light at 0700 h. All animals were tested during

the light phase. Rats were housed individually in standard cages (16.5 in × 8.5 in × 8 in) with ad lib access to food and water and were acclimated upon arrival for at least 5 days prior to experimentation. All experiments were carried out in accordance with the Institutional Animal Care and Use Committee of the East Orange, New Jersey Health Care System, Veterans Affairs Medical Center.

Surgery

Sprague Dawley and WKY rats were anesthetized with Nembutal (50 mg/kg i.p.), and supplemented as necessary. Guide cannulas (26 g, Plastics One, Roanoke, VA, USA) were implanted bilaterally (4 mm posterior and 2.5 mm lateral from bregma, and −3.1 mm ventral from brain surface) directed at the dentate gyrus region of the hippocampus. Each guide cannula was fixed to skull screws (stainless steel) using dental acrylic cement. A stylet was inserted into the guide cannula to keep the cannula patent.

Electrodes were implanted into the periorbital muscles for eyeblink conditioning. Four Teflon-coated, stainless steel wires (75 µm diameter, AM Systems) had the insulation stripped from one end that was inserted into the muscle. The other end of the wire was inserted into a plastic connector (Cannon Centi-loc, ITT Cannon, Santa Ana, CA) that was glued to three to four skull screws using dental acrylic. Two wires were used to record electromyography (EMG) and the other two wires delivered electrical stimulation.

Following the surgical procedure, sutures were used as needed and rats were post-operatively treated with flunixin meglumine (2.5 mg/kg, s.c.) for 2 days. Rats were allowed at least 4 days to recover from surgery.

Classical Conditioning of the Eyeblink Response

Eyeblink conditioning was conducted in a sound-attenuated chamber (27 cm × 29 cm × 43 cm) with a viewing window (Med Associates, St. Albans, VT, USA). The EMG signals were recorded from electrodes that were connected to a differential AC amplifier through a cable attached to the plastic connector on the rat's head. EMG signals were filtered (300–500 Hz) and amplified (10,000X, A-M Systems Model 1700, Everett, WA, USA). Electrical stimulation of the periorbital muscles was delivered by a stimulus isolation unit (Coulbourn Instruments, Whitehall, PA, USA). A computer equipped with an A/D board and LabView software (National Instruments, Austin, TX, USA) controlled stimuli presentation and recording of EMG signals digitized at a sampling rate of 1000 Hz. One day prior to conditioning, freely moving rats were habituated to the apparatus for 30 min. During habituation, EMG signal quality was determined. Rats were conditioned for 1 or 2 days following habituation.

Rats were conditioned using a delay conditioning paradigm. Rats received 100 conditional stimulus (CS)-unconditional stimulus (US) paired trials per day. An auditory stimulus (500 ms, 82 dB white noise, 10 ms rise/fall) served as the CS. Electrical stimulation of the periorbital muscles (10 V, 10 ms) served as the US. CS and US co-terminated. The inter-trial interval (ITI) ranged from 15 to 35 s with an average of 25 s.

Electromyography was analyzed to determine the occurrence of eyeblinks using a custom designed script in S-Plus (version 6.1, Insightful Corporation, Seattle, WA, USA). For each trial, the 250 ms prior to the presentation of the CS was used as a baseline for each trial. An eyeblink, conditioned response (CR), was designated when the EMG activity exceeded a threshold amplitude following the CS onset and prior to the US onset. Threshold amplitude was equal to the mean amplitude of the baseline plus four standard deviations of the baseline activity. Any response recorded during the first 30 ms of the CS onset (250–280 ms) was not counted as a CR, as this time frame typically indicates an orienting response and represents less than 10% of eyeblinks. To evaluate the rate of acquisition, trials were grouped into five blocks of 20 trials per day. Analysis of variance (ANOVA) with repeated measures was used to analyze CR.

BDNF Administration

For animals receiving infusions prior to eyeblink conditioning, an infusion cannula (33 g, Plastics One, Roanoke, VA, USA) attached to a Hamilton syringe via polyethylene tubing (PE 50, Becton Dickinson, Sparks, MD, USA) was inserted into the guide cannula. Sterile saline (0.5 µl) or rhBDNF (0.5 µg/0.5 µl; R&D Systems, Minneapolis, MN) was administered (0.1 µl/min) into the dentate gyrus region of the hippocampus. After drug administration, the infusion cannula was allowed to remain in place for 5 min, and then removed and replaced with a stylet. Infusions were given approximately 45 min (40–50 min range) prior to the start of the eyeblink conditioning session. Saline or BDNF was infused prior sessions 1 and 2 of conditioning.

Tissue Extraction

Animals for RT-PCR analysis were sacrificed and the hippocampus was extracted approximately 1 h after Day 1 of eyeblink conditioning. Because BDNF levels fluctuate throughout the day, tissue collection was confined to 3 h after the onset of the light cycle, approximately between 10:00 a.m. and 1:00 p.m. After decapitation and rapid removal of the brain, CA1, CA3, and dentate gyrus regions of both hippocampi were dissected rapidly on ice, placed in microcentrifuge tubes, and stored in dry ice. Net wet tissue weight of the tissue was recorded. Samples were stored at −80°C pending analysis.

RT-PCR

mRNA for BDNF, TrkB (high affinity BDNF receptor), and the immediate early gene Arc (activity-regulated cytoskeleton-associated protein) was measured using RT-PCR. Total RNA was isolated from the dentate gyrus by submerging in Trizol reagent and adding Zirconium disruption beads (Thomas Scientific, Swedesboro, NJ, USA). Supernatant was further processed and DNase treated as per manufacturer's instructions (Direct-zol RNA mini-prep, Zymo Research, Irvine, CA, USA). The RNA concentration was quantified using the NanoDrop Spectrophotometer (NanoDrop, Wilmington, DE, USA). Total RNA was reverse transcribed by first denaturing 1 µg sample and 1 µl of 300 ng/µL RT primer at 65°C for 5 min and then chilling on ice. Next, 6 µl of 5× Superscript Buffer, 1.5 µl 0.1M DTT, 1.5 µl 10 mM dNTPs, 1 µl Superase In, and 1 µl Superscript III (Life Technologies, Invitrogen, Carlsbad, CA, USA) were added to the samples and incubated at 25°C for 10 min, followed by 45°C for 2 h. The RT reaction was terminated by heating at 70°C for 15 min and

the cDNA stored at −20°C. RT-PCR was performed using Roche Lightcycler® containing 3 µl of cDNA, 10 µl Taqman Universal PCR master mix, 1 µl of Taqman probe (Bdnf Taqman Probe, Rn02531967; Ntrk2 Taqman Probe, Rn01441749_ml; Arc Taqman Probe, Rn00571208_gl; 18S Taqman Probe, hs99999901_s1; Applied Biosystems, Grand Island, NY, USA), 1 µl of Bovine Serum Albumin (2.5 mg/mL; BioFire, Salt Lake City, UT, USA), and 5 µl dH_20.

The cycle threshold (CT) value was determined for each probe. Data for each target gene were assayed in duplicate and averaged, target values were normalized to the mean of the housekeeping gene 18S ribosomal RNA, which showed the lowest amount of variability across strain and treatment. Fold differences between samples for each gene product were calculated as follows:

$$\frac{2^{\wedge}\text{(Sample with highest CT value for target gene-individual sample's CT value for target gene)}}{2^{\wedge}\text{(Sample with highest CT value for 18S rRNA-individual sample's CT value for 18S rRNA)}}$$

Statistical Analysis

Statistical analyses were conducted using Statistical Package for the Social Sciences (SPSS for Windows, Version 16, SPSS, Inc., Chicago, IL, USA). All results were considered significant at $\alpha = 0.05$. Behavioral data for mRNA analysis were evaluated with a mixed design ANOVA for CR probability with blocks as a within-subject factor and strain as a between-subject factor. Average CR probability was calculated for blocks consisting of 20 trials, resulting in five blocks per session. Behavioral data for BDNF administration had a similar experimental design, but with the addition of treatment as a between subjects factor. mRNA data were analyzed using an ANOVA with strain and conditioning as between subjects factors. Separate analyses were conducted for BDNF, TrkB, and Arc mRNA in each hippocampal subregion. Only significant ($p < 0.05$) and trending ($p < 0.1$) results are reported.

Results

Learning-Induced Changes in Hippocampal BDNF, TrkB, and Arc mRNA

Behavior

Sprague Dawley ($n = 7$) and WKY ($n = 8$) rats were trained in one session of delay classical conditioning of the eyeblink response followed by sacrifice for assessment of hippocampal BDNF, TrkB, and Arc mRNA. Due to problems with EMG recording, 1 SD and 2 WKY rats could not be evaluated for behavior; these rats showed clear eyeblink to periorbital electrical stimulation US and should demonstrate classical conditioning similar to other rats. Therefore, all rats were included in the mRNA analysis. Acquisition of classical conditioning was significantly faster and performed to a greater degree in WKY rats compared to SD rats, main effect of strain [$F(1, 10) = 5.02$, $p < 0.05$] (**Figure 1**), replicating previous results (45). Overall, general learning was demonstrated by a main effect of block [$F(4, 40) = 8.38$, $p < 0.001$]. No interaction between block and strain was observed. Ninety to one hundred and twenty minutes following the conditioning sessions, rats were sacrificed and the hippocampus removed, subdivided, and stored for subsequent analysis by qRT-PCR.

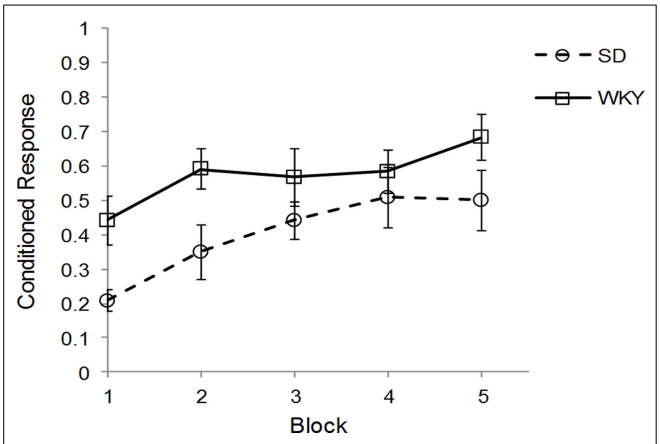

FIGURE 1 | Strain differences in classical eyeblink conditioning. Wistar-Kyoto (WKY) and Sprague Dawley (SD) rats were trained in delayed classical conditioning of the eyeblink response. A session consisted of five blocks of 20 trials. WKY rats acquired eyeblink conditioning significantly faster and to a greater extent than SD rats, as demonstrated by higher levels of conditioned responses.

Brain-Derived Neurotrophic Factor

In the DG, learning increased BDNF mRNA in SD to a greater extent than WKY, as demonstrated by a strain × conditioning interaction [$F(1,19) = 5.06$, $p < 0.05$] (**Figure 2**). BDNF mRNA was increased by conditioning, main effect of conditioning [$F(1,19) = 15.4$, $p < 0.001$], and both strains showed learning-induced increases [SD: $t(9) = 3.17$, $p < 0.05$; WKY: $t(10) = 2.4$, $p < 0.05$]. In CA3, conditioning enhanced BDNF mRNA [$F(1,20) = 12.94$, $p < 0.005$] with a trend for upregulation in CA1 [$F(1,19) = 3.57$, $p = 0.074$], but these changes did not differ between strains.

TrkB Receptor

In all three subregions of the hippocampus, rats in the classical conditioning group had higher TrkB mRNA than sham rats [DG: $F(1,19) = 8.09$, $p < 0.01$; CA3: $F(1,20) = 10.32$, $p < 0.005$; CA1: $F(1,20) = 6.24$, $p < 0.05$] (**Figure 2**). However, TrkB mRNA did not differ between strains in any of the hippocampal subregions.

Arc

In the DG, classical conditioning upregulated Arc mRNA [$F(1,19) = 4.67$, $p < 0.05$] (**Figure 2**). Conditioning increased Arc mRNA to a greater extent in SD rats compared to WKY rats, main effect of strain [$F(1,19) = 4.94$, $p < 0.05$], strain × conditioning interaction [$F(1,19) = 3.04$, $p = 0.097$]. Arc mRNA did not differ between strains or conditioning groups in the CA1 and CA3 regions.

Effects of Intrahippocampal BDNF on Delay Eyeblink Conditioning Acquisition

Following CCER, up-regulation of BDNF and Arc mRNA in the DG was blunted in the WKY rats compared to SD rats. Therefore, the effects of administering BDNF into the DG at

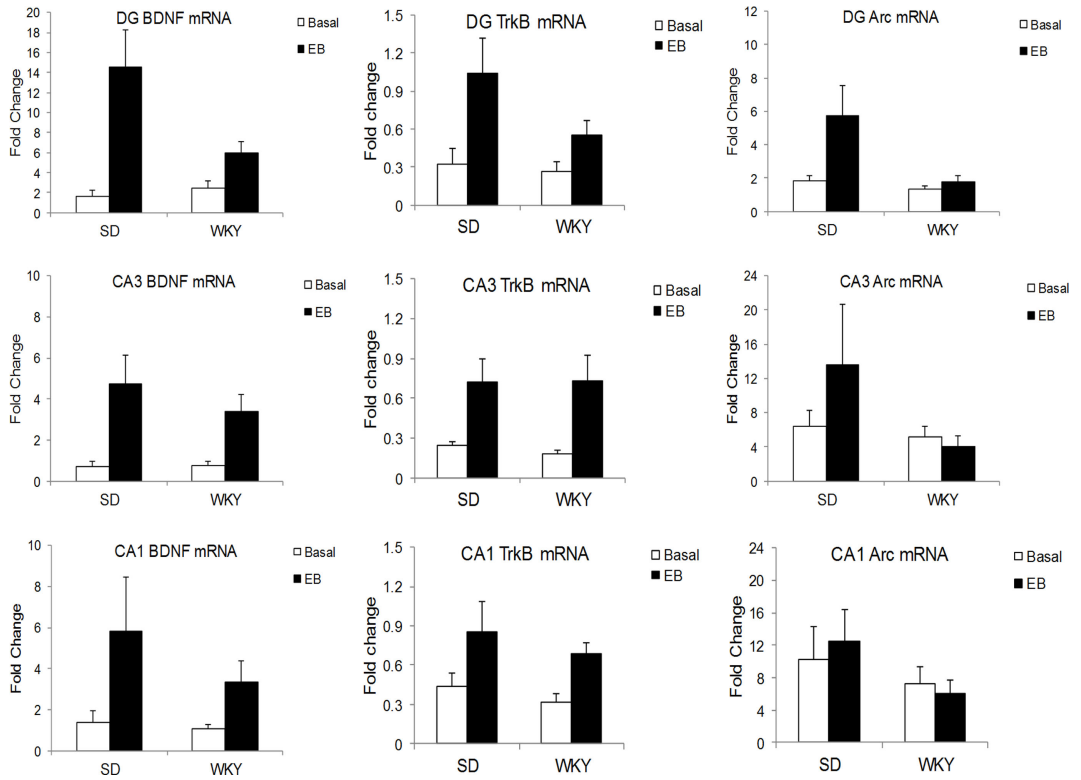

FIGURE 2 | Learning-induced increases in BDNF, TrkB, and Arc mRNA differed between SD and WKY rats. Following a single session of classical conditioning of the eyeblink response (CCER), the hippocampal subregions were dissected, and BDNF (left), TrkB (middle), and Arc (right) mRNA was assessed in the dentate gyrus (upper), CA3 (middle), and CA1 (lower) subregions of the hippocampus. BDNF mRNA was significantly increased following acquisition of CCER in the dentate gyrus and CA3. In CA1, the main effect of learning did not reach significance ($p = 0.074$). Moreover, learning-induced changes in the dentate gyrus of WKY rats were significantly smaller than that in SD rats. By contrast, learning-induced changes of BDNF mRNA in CA3 were similar between strains. Learning caused increases of TrkB mRNA in all hippocampal subregions and increases were similar between SD and WKY rats. Finally, acquisition of CCER increased Arc mRNA only in the dentate gyrus, but not CA3 or CA1. The changes in Arc mRNA in the dentate gyrus were significantly smaller in the WKY rat compared to SD rat.

the time of CCER were evaluated in both strains. Rats (SD-saline, $n = 7$; SD-BDNF, $n = 8$; WKY-saline, $n = 9$; WKY-BDNF, $n = 9$) were administered and conditioned in two sessions. Only animals that had reliable EMG signals on both days of training were used in the analysis. CRs increased as a consequence of training in all rats for days 1 and 2, main effect of block [$F(4, 116) = 8.148$, $p < 0.001$] and main effect of day [$F(1, 29) = 24.28$, $p < 0.001$] (**Figure 3**). Similar to previous studies, WKY rats acquired faster than SD rats, main effect of strain [$F(1, 29) = 12.48$, $p < 0.001$]. Importantly, BDNF infusion into the DG affected WKY but not SD rats, strain × treatment interaction [$F(1, 29) = 4.972$, $p < 0.05$]. Neither the main effect of treatment nor the block × strain × treatment interaction was significant.

Given the significant strain × treatment interaction, further analysis was conducted on the effects of BDNF in each strain. In SD rats, BDNF treatment did not alter the acquisition of delay CCER, as neither the main effect nor interactions involving treatment were significant. By contrast, WKY rats were significantly slowed in acquisition by BDNF administration, main effect of treatment [$F(1, 16) = 8.7$, $p < 0.01$] and treatment × day × block interaction [$F(4, 64) = 2.72$, $p < 0.05$].

Discussion

The present study utilized the WKY rat to investigate the role of hippocampal BDNF in the facilitated associative learning that is observed in behaviorally inhibited individuals. The hippocampus was the focus of this study because it contains a high amount of BDNF (5–8), and dysfunction of hippocampus and BDNF systems both represent vulnerabilities for developing anxiety disorders (2, 9, 10, 20, 29). Furthermore, hippocampal damage leads to facilitated acquisition of delay CCER (39), similar to high behaviorally inhibited humans (41–44) and animals (45). In agreement with previous findings, the present study found WKY rats acquired delay CCER faster and to a greater degree than SD rats. Acquisition of CCER was associated with increased BDNF and Arc mRNA in the DG and CA3 of the hippocampus. Importantly, WKY rats had smaller increases than SD rats in the DG. TrkB mRNA was also increased following CCER in all hippocampal subregions, but these changes did not differ between strains. The smaller learning-induced changes of BDNF and Arc mRNA in WKY rats suggested that the lack of BDNF and resultant hippocampal dysfunction in this rat strain may be responsible for facilitated CCER. To

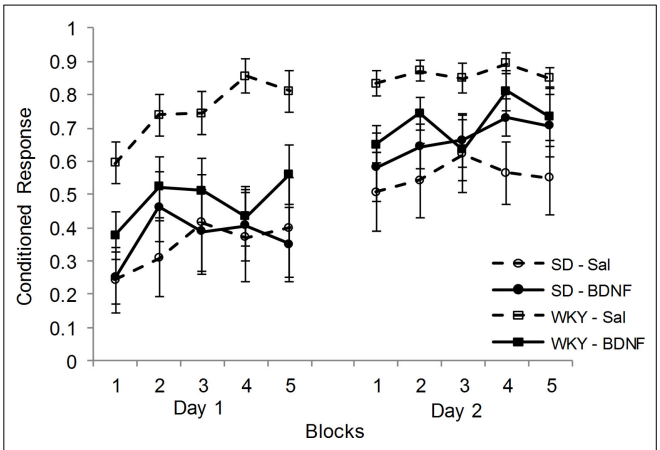

FIGURE 3 | Intrahippocampal BDNF normalized classical conditioning of the eyeblink response in the WKY rat. BDNF was administered into the dentate gyrus of SD and WKY rats prior to each of two sessions of eyeblink conditioning. Saline-treated WKY rats acquired eyeblink conditioning significantly faster and to a greater extent than saline-treated SD rats, as demonstrated by more conditioned responses. BDNF administration in WKY rats slowed classical eyeblink conditioning to a level similar to that observed in SD rats. BDNF treatment did not alter classical conditioning in SD rats.

test this hypothesis, exogenous BDNF was administered into the DG of SD and WKY rats prior to eyeblink conditioning sessions. Intrahippocampal BDNF slowed CCER acquisition of WKY rats to a level similar to SD rats. By contrast, BDNF infusions did not alter CCER acquisition in SD.

Brain-derived neurotrophic factor is important for hippocampal-dependent learning (61, 62). With respect to classical conditioning, contextual fear conditioning enhanced the number of CA1 neurons expressing BDNF immunoreactivity (63). BDNF heterozygous knockout mice were poorer in acquiring contextual but not cued fear conditioning, suggesting a differential action of BDNF on hippocampal-dependent and -independent forms of classical conditioning (64). In the present study, acquisition of a hippocampal-independent form of CCER increased BDNF mRNA in all three subregions of the hippocampus.

An increase in BDNF causes somatodendritic expression of Arc mRNA in the dentate gyrus (65). Arc, an immediate early gene, is one of the first genes transcribed after receiving extracellular signaling and is implicated in learning and memory. The induction of Arc enlarges dendrites, impacts dendritic structure and organization, is activated in dendrites in an NMDA-dependent manner (66), and is increased several hours post-BDNF infusion (67). Arc was increased in the hippocampus following hippocampal-dependent trace and contextual fear conditioning, but not after hippocampal-independent delay fear conditioning (68). The lack of change in Arc following hippocampal-independent delay fear conditioning contrasts with results of the present study, which showed increases in Arc mRNA following hippocampal-independent delay CCER. In the present study, the increase in Arc mRNA was only observed in the DG and not in the other hippocampal subregions. Therefore, the lack of change in Arc following fear conditioning may be due to dilution of the Arc changes in DG by other hippocampal subregions, although differences between fear conditioning and CCER cannot be entirely ruled out either.

Although WKY rats acquired delay CCER faster and to a greater extent than SD rats, they had smaller increases in BDNF and Arc mRNA than SD rats. Blunted changes in BDNF and Arc mRNA observed in WKY rats can be interpreted as poorer hippocampal function, and is supported by impaired hippocampal synaptic plasticity in WKY rats (56). Thus, our results support the view that damage or dysfunction of the hippocampus can lead to better acquisition of delay CCER (39).

Brain-derived neurotrophic factor administration enhances various forms of learning and memory (62). Infusion of BDNF into the hippocampus enhanced water maze reversal learning and reduced anxiety-like behavior in an elevated plus maze, suggesting that hippocampal BDNF improve hippocampal-dependent learning and reduce anxiety (69). Additionally, hippocampal infusions of BDNF enhanced contextual fear conditioning in BDNF heterozygous knockout mice (64) and transgenic mice expressing active CREB or their wild-type counterparts (70). While most evidence is that BDNF enhances hippocampal-dependent forms of learning, the effect of hippocampal BDNF administration on hippocampal-independent learning has not been addressed. The present study shows that administration of BDNF into the hippocampus of WKY rats slowed acquisition of delay CCER to a level equivalent to that demonstrated by SD rats. Thus, hippocampal BDNF administration can result in poorer acquisition on some forms of learning and in some rat strains. In this regard, the delay CCER paradigm may be a special case because hippocampal damage can facilitate acquisition (39).

The results of the present study provide a potential link between three anxiety vulnerabilities: BDNF dysfunction, small hippocampal volume and impaired function, and behavioral inhibition. BDNF dysfunction can lead to reduced hippocampal volume and impaired hippocampal-dependent learning. In humans, abnormally low levels of BDNF are associated with a smaller hippocampal volume (22). However, the effect of the BDNF Val66Met SNP on hippocampal volume in humans is unclear (71), although an association between reduced hippocampal volume and the interaction of Val66Met SNP with environmental factors (childhood maltreatment) is growing (72, 73). Individuals with the Val66Met SNP have impairments in learning and memory that are generally considered to be hippocampal dependent (74). Mice with the Val66Met SNP have smaller hippocampi, reduced activity-dependent secretion of BDNF, dendritic shrinkage in the DG, and impaired extinction of fear conditioning compared to wild-type mice (9, 29). It is possible that BDNF and hippocampal dysfunction represent the same vulnerability. Early childhood trauma or chronic stress is a risk factor for anxiety disorders. One of the structures most affected by chronic stress is the hippocampus, due to the density of glucocorticoid receptors (GRs) and its involvement in regulating the HPA axis (75–77). One mechanism by which stress has a negative impact on hippocampal morphology and function is by decreasing hippocampal BDNF, resulting in decreased neurogenesis, dendritic atrophy, and impaired cognition (3, 4, 28, 78–80). These stress-induced reductions of BDNF may relate to the reductions of BDNF protein and hippocampal volume in some patients without Val66Met genotype.

While there is an abundance of evidence associating BDNF and hippocampus volume and function, links between inhibited temperament and BDNF or hippocampal dysfunction has been sparse. Individuals with inhibited temperament have abnormal hippocampal processing of novel stimuli in humans (37, 81). Interestingly, activation of the hippocampus to novel faces was most strongly associated with inhibited temperament *and* childhood maltreatment (37). As described above, childhood maltreatment and chronic stress are associated with smaller hippocampal volume and hippocampal dysfunction. In animal studies, the behaviorally inhibited WKY rat has a smaller hippocampus than non-inhibited rat strains (56), impaired hippocampal synaptic plasticity (56), and poorer performance on hippocampal-dependent learning procedures (49, 57). The WKY rat also behaves similarly to rats with hippocampal damage (56, 58). Thus, there is little evidence to link inhibited temperament with smaller hippocampus or BDNF dysfunction, except for the animal work. However, inhibited temperament may interact with either BDNF/hippocampal dysfunction to exacerbate vulnerability to develop anxiety disorders.

In summary, BDNF dysfunction in the hippocampus was observed in an animal model of behavioral inhibition, the WKY rat. This dysfunction was related to facilitated acquisition of hippocampal-independent associative learning. Gain of function experiments by administering BDNF into the hippocampus of WKY rats "normalized" associative learning. The results suggest a possible mechanism by which hippocampal dysfunction and behavioral inhibition leads to pathological associative learning and vulnerability to develop anxiety disorders.

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Autism and Classical Eyeblink Conditioning: Performance Changes of the Conditioned Response Related to Autism Spectrum Disorder Diagnosis

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Changes in the timing performance of conditioned responses (CRs) acquired during trace and delay eyeblink conditioning (EBC) are presented for diagnostic subgroups of children having autism spectrum disorder (ASD) aged 6–15 years. Children diagnosed with autistic disorder (AD) were analyzed separately from children diagnosed with either Asperger's syndrome or Pervasive developmental disorder (Asp/PDD) not otherwise specified and compared to an age- and IQ-matched group of children who were typically developing (TD). Within-subject and between-groups contrasts in CR performance on sequential exposure to trace and delay EBC were analyzed to determine whether any differences would expose underlying functional heterogeneities of the cerebral and cerebellar systems, in ASD subgroups. The EBC parameters measured were percentage CRs, CR onset latency, and CR peak latency. Neither AD nor Asp/PDD groups were impaired in CR acquisition during trace or delay EBC. Both AD and Asp/PDD altered CR timing, but not always in the same way. Although the AD group showed normal CR timing during trace EBC, the Asp/PDD group showed a significant 27 and 28 ms increase in CR onset and peak latency, respectively, during trace EBC. In contrast, the direction of the timing change was opposite during delay EBC, during which the Asp/PDD group showed a significant 29 ms decrease in CR onset latency and the AD group showed a larger 77 ms decrease in CR onset latency. Only the AD group showed a decrease in CR peak latency during delay EBC, demonstrating another difference between AD and Asp/PDD. The difference in CR onset latency during delay EBC for both AD and Asp/PDD was due to an abnormal prevalence of early onset CRs that were intermixed with CRs having normal timing, as observed both in CR onset histograms and mean CR waveforms. In conclusion, significant heterogeneity in EBC performance was apparent between diagnostic groups, and this may indicate that EBC performance can report the heterogeneity in the neurobiological predispositions for ASD. The findings will inform further explorations with larger cohorts, different sensory modalities, and different EBC paradigms and provide a reference set for future EBC studies of children having ASD and non-human models.

Keywords: autism, eyeblink conditioning, timing, cerebellum, diagnostic specificity

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INTRODUCTION

The purpose of this paper is to describe the changes in conditioned response (CR) performance during trace and delay eyeblink conditioning (EBC) in a cohort of high-functioning children having autism spectrum disorder (ASD). The *de novo* acquisition and mean parameters of CR timing of this cohort of 14 children with ASD and 16 typically developing (TD) children were reported by Oristaglio et al. (1). Here, we provide more detailed information regarding the distribution of CR performance changes in the cohort described by Oristaglio et al. (1), re-grouped by ASD-spectrum diagnosis in order to determine whether heterogeneity in EBC performance may relate to diagnostic category within a high-functioning group of children. Our experimental design sequentially employed trace and delay EBC paradigms in every subject to provide within-subject and between-group contrasts of EBC performance on two paradigms that differ in their degree of cerebral involvement (2).

Two previous studies (1, 3) of subjects having idiopathic ASD demonstrated changes in CR timing during delay EBC without a reduction in the rate of CR acquisition, agreeing that CR timing is shifted earlier with ASD. In a complementary pair of studies (4, 5), delay EBC in children and adults with Fragile X, a severe form of intellectual disability in which some individuals have comorbid symptoms of ASD, showed reduced CR acquisition and earlier CR peak latencies with greater impairments in adults as compared to children. Thus, four studies have demonstrated heterogeneity in the changes in EBC performance among individuals with varying ASD symptomatology. Although not explicitly emphasized, this heterogeneity is mirrored in mouse models of idiopathic and syndromic ASD in which there are differential changes in the rate of CR acquisition and the directionality of CR timing that depend on the specific gene mutation induced (4, 6, 7).

For our study, the rationale for using a sequence of trace and delay EBC was to provide the first examination of trace EBC in children with ASD, followed by an opportunity to determine whether we could replicate the previous finding of mistimed CRs during delay EBC. Although the division of the original cohort in Oristaglio et al. (1) into subgroups necessarily reduces group size and statistical power, our goal is to provide these data so that they may serve as a reference for future studies of EBC using larger cohorts of children having ASD with varying degrees of functional impairment and for those that use classical conditioning of the eyeblink or other responses in non-human animal models of ASD.

MATERIALS AND METHODS

Subjects

The subjects were 30 children (age 6–15 years) recruited at the Drexel Autism Center at Friends Hospital in Philadelphia. The study was approved by the IRB of the Drexel University College of Medicine and a legal custodian of the subjects signed a consent form prior to participation. Fourteen subjects were diagnosed with ASD (13 males, 1 female), and 16 were typically developing (TD; 7 males, 9 females). ASD subjects included those diagnosed with autistic disorder (AD; $n = 7$), Asperger's disorder (Asp, $n = 2$), and pervasive developmental disorder-not otherwise specified (PDD-NOS, $n = 5$) based on the content-area scores on the revised Autism Diagnostic Interview [ADI-R; Ref. (8)] and the Childhood Autism Rating Scale (9). By convention, children with Asp or PDD-NOS met criteria on two of the three domains assessed on the ADI-R and showed developmental delays prior to 3 years of age based on retrospective report. Exclusion factors for the ASD group were the presence of psychiatric diagnosis including Rett's disorder or childhood disintegrative disorder. TD subjects had no psychiatric diagnoses other than one diagnosed with oppositional defiant disorder and obsessive-compulsive disorder. The ASD and TD subjects were approximately matched for age [mean ± 1 SEM: TD, 9.6 ± 2.5; AD, 7.7 ± 1.3; Asp/PDD, 9.4 ± 0.6 years; $F(2,27) = 1.4$, $p = 0.3$] and IQ [TD, 111 ± 3; AD, 107 ± 3; Asp/PDD, 104 ± 5 WASI score; $F(2,27) = 0.8$, $p = 0.5$].

Eyeblink Conditioning

Eyeblink conditioning was carried out as specified in Oristaglio et al. (1). Briefly, the subjects watched a silent movie while they wore headphones that delivered tones binaurally. Eye blinks were detected by an infrared emitter-sensor approximately 1 cm from the right eye. Eye blinks were defined as a change in sensor output greater than 15 SD above the mean baseline. The conditioned stimulus (CS) was a 1-kHz, 61-dB tone. The unconditioned stimulus (US) was a 100 ms puff of air (5 psi source) delivered to the eye through a tube (1 mm i.d.) attached to the sensor. EBC sessions contained 90 trials divided into nine blocks of 10 trials. The first nine trials in each block consisted of paired CS–US trials, and the 10th was a CS-alone trial. The intertrial interval was 20 s (range 15–25 s). Three EBC sessions occurred on separate visits. The first two sessions consisted of trace EBC, which was performed using a 200-ms CS, a 500-ms trace interval, and then the US (700 ms CS–US interval). The third session consisted of delay EBC also at the 700 ms CS–US interval and was performed by extending the CS duration so that it coterminated with the US. CRs were defined as eye blinks that occurred at least 80 ms after CS onset and prior to US onset. This experimental design used a constant CS–US interval in order to place no explicit motor demand on CR timing while changing only the EBC paradigm from trace to delay on session 3. Although the two paradigms would necessarily interact, there is precedent for using sequences of delay and trace EBC within the same human subjects in clinical studies (10, 11). Moreover, functional brain imaging has demonstrated differential brain activation in humans experiencing both paradigms concurrently (2).

The mean time between the two trace EBC sessions was 13 ± 2 days, and the mean time between the second trace EBC session and the delay EBC session was 27 ± 9 days. There was not a difference between the groups in the number of days between sessions for between the trace sessions [$F(2,27) = 1.7$, $p = 0.2$] or between the trace and delay sessions [$F(2,27) = 1.0$, $p = 0.4$].

Statistical Analysis

Conditioned response acquisition was examined using mixed effects analysis of variance. Differences in CR performance were examined with three methods using the post-CS response

distributions as primary data. First, differences in the means of the individual responses were evaluated by paired *t*-test. Second, differences in CR distributions were evaluated using the non-parametric, two-sample Kolmogorov–Smirnov (K–S) test, a highly liberal test that evaluates whether there is a difference in the shape of the cumulative probability function at any unspecified location in the CS–US interval. Third, differences between medians were evaluated using the non-parametric Mood's median test. Mood's median test evaluated whether there is a difference in the ratio of responses below and above an aggregate median, which is tested using a 2 × 2 contingency table. Mood's median test was chosen due to its robustness against differences in the shapes of the latency distributions between groups. The three tests were employed under the rationale that the most significant effects of ASD diagnosis would produce a significant change not only in the cumulative probability function but also in the more conservative mean and median tests that inform about the directionality of a change in central tendency. We labeled CR performance changes as strong or moderate depending on the number of statistical tests that detected a difference between the distributions. CR waveform analysis was carried out by averaging CRs triggered on CS onset. Last, mean CR onset and peak latency for each individual subject was *z*-transformed using the TD mean and SD and presented for each EBC session. The threshold used for statistical significance was 5%. Data are presented as the mean ± 1 SEM.

RESULTS

As reported for this cohort (1), the rate of CR acquisition and asymptotic percentage CRs did not differ between the 14 ASD and 16 TD subjects during any of the three EBC sessions. **Figure 1** shows the percentage CRs over sessions in which the ASD cohort was divided into diagnostic subgroups. All groups showed associative learning by displaying a significant increase in percentage CRs from the first to the second session [$F(1,17) = 3.1$, $p < 0.005$]. There was not a significant difference in learning rate between the groups [$F(1,27) = 0.01$, $p = 0.99$] nor a significant difference in the shape of the learning curves across the trace EBC sessions [$F(1,17) = 0.8$, $p = 0.67$; **Figure 1A**]. Switching to delay EBC did not significantly change the percentage CRs from the previous session (**Figure 1B**), and there was no significant difference between groups as the overall mean CR frequency during delay EBC was 48 ± 4, 41 ± 7, and 46 ± 8% for TD, AD, and Asp/PDD, respectively [$F(2,27) = 0.1$, $p = 0.93$ for the first three blocks; $F(8,216) = 0.5$, $p = 0.63$ for all nine blocks]. Thus, there was no indication that AD or Asp/PDD impaired the ability to acquire CRs during trace or delay EBC or show asymptotic percentage CRs characteristic of the TD group under these EBC parameters.

Figure 2 shows post-CS time histograms and cumulative probability plots of CR onset and peak latency over two sessions of trace EBC. The mean CR onset latencies were: TD, 441 ± 5; AD, 441 ± 7; Asp/PDD, 468 ± 7 ms. *T*-tests did not indicate a difference between TD and AD [$t(1,748) = 0.09$, n.s.] but did indicate a significant difference between TD and Asp/PDD [$t(1,735) = 3.1$, $p < 0.01$]. K–S tests indicated that the shape of both the AD ($D = 0.04$, $p < 0.01$) and Asp/PDD ($D = 0.08$, $p < 0.001$) distributions differed from TD. However, Mood's median test did not detect a significant difference of the median CR onset latency of either diagnostic group when compared to TD (both $p \geq 0.1$).

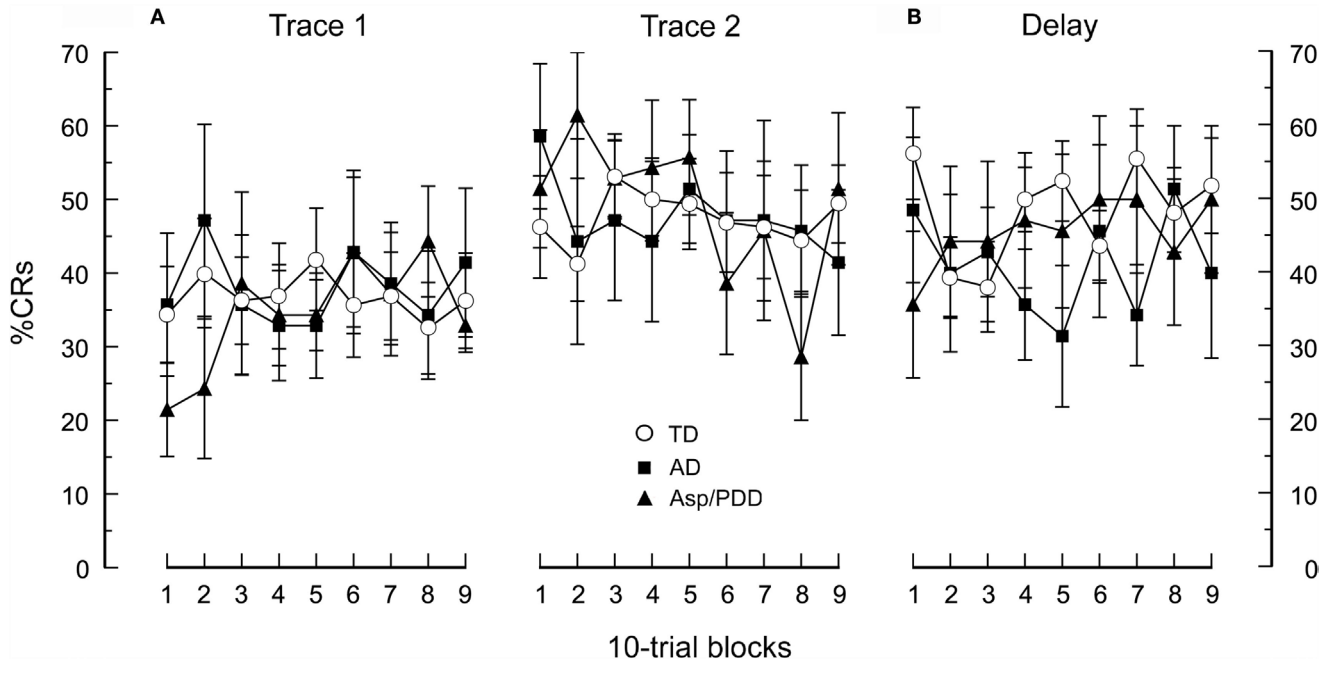

FIGURE 1 | CR acquisition for the AD, Asp/PDD, and TD groups. **(A)** During two sessions of trace EBC. **(B)** During a subsequent session of delay EBC. Mean data are presented in 10-trial blocks. Error bars show ± 1 SEM. Curves showing TD are taken from Oristaglio et al. (1).

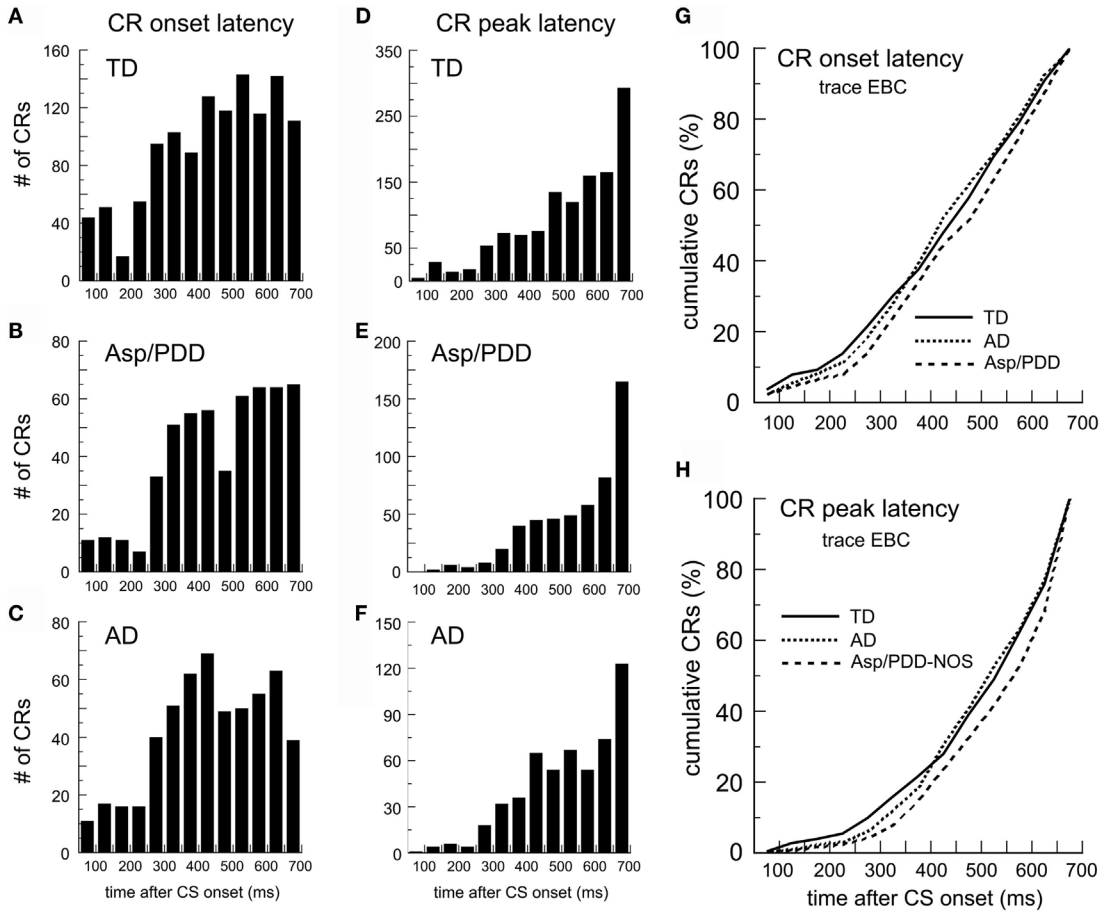

FIGURE 2 | **CR timing during trace EBC**. **(A–C)** Post-CS time histograms of CR onset for the TD **(A)**, Asp/PDD **(B)**, and AD **(C)**. **(D–F)** CR peak latency histograms from the same groups. **(G,H)** Cumulative probability plots of CR onset and CR peak. Bin width = 50 ms. Time 0 = CS onset.

Thus, there was a moderate indication of a small delay in CR onset latency during trace EBC for Asp/PDD (27 ms), but not AD.

The mean CR peak latencies during trace EBC were TD, 508 ± 4; AD, 518 ± 6; and Asp/PDD, 536 ± 6 ms. Again, *t*-tests did not indicate a difference between TD and AD [$t(1,643) = 1.31$, n.s.], but did indicate a significant difference between TD and Asp/PDD [$t(1,592) = 3.6$, $p < 0.01$]. K–S tests indicated that the shape of both the AD ($D = 0.04$, $p < 0.05$) and Asp/PDD ($D = 0.09$, $p < 0.01$) distributions differed from TD. However, Mood's median test did not detect a significant difference between either diagnostic group and TD, although Mood's test between TD and Asp/PDD was nearly significant ($X^2 = 3.79$, $p = 0.052$). Thus, as for CR onset latency, there was a moderate indication of a small increase in CR onset latency during trace EBC for Asp/PDD (28 ms), but not for AD.

Figure 3 shows the identical analysis for delay EBC. The mean CR onset latencies were TD, 472 ± 7; AD, 395 ± 13; and Asp/PDD, 443 ± 11 ms. *T*-tests indicated a highly significant difference between TD and AD [$t(617) = 5.4$, $p < 0.001$] and a significant difference between TD and Asp/PDD [$t(633) = 2.2$, $p < 0.05$]. The CR onset latency histogram of the TD group was shaped such that the prevalence of CR onsets increased as the CS–US interval progressed, reaching maximum 650 ms after CS onset (**Figure 3A**, arrow). In contrast, AD subjects showed a mode CR onset at 300 ms, and the majority of their CRs were initiated between 200 and 400 ms after CS onset (**Figure 3C**, asterisks). The distribution of the Asp/PDD group had two peaks, the first identical to AD between 200 and 400 ms (**Figure 3B**, asterisks) and the second close to the TD peak at 600 ms (**Figure 3B**, arrow). K–S analyses indicated that the changes in distribution shapes were highly significant (AD vs. TD: $D = 0.21$, $p < 0.001$; Asp/PDD vs. TD, $D = 0.11$, $p < 0.001$). Mood's median test detected a highly significant difference between the CR onsets of TD and AD ($X^2 = 20.8$, $p < 0.001$) but not between TD and Asp/PDD ($X^2 = 3.3$, n.s.). Thus, there was a strong indication of a large decrease in CR onset latency for AD (77 ms) and a moderate indication for a smaller decrease for Asp/PDD (29 ms).

The mean CR peak latencies during delay EBC were: TD, 544 ± 13; AD, 482 ± 11; Asp/PDD, 536 ± 10 ms. *T*-tests indicated a highly significant difference between TD and AD [$t(282) = 3.6$, $p < 0.001$], but not between TD and Asp/PDD [$t(298) = 0.4$, n.s.]. K–S analysis indicated a highly significant difference between TD

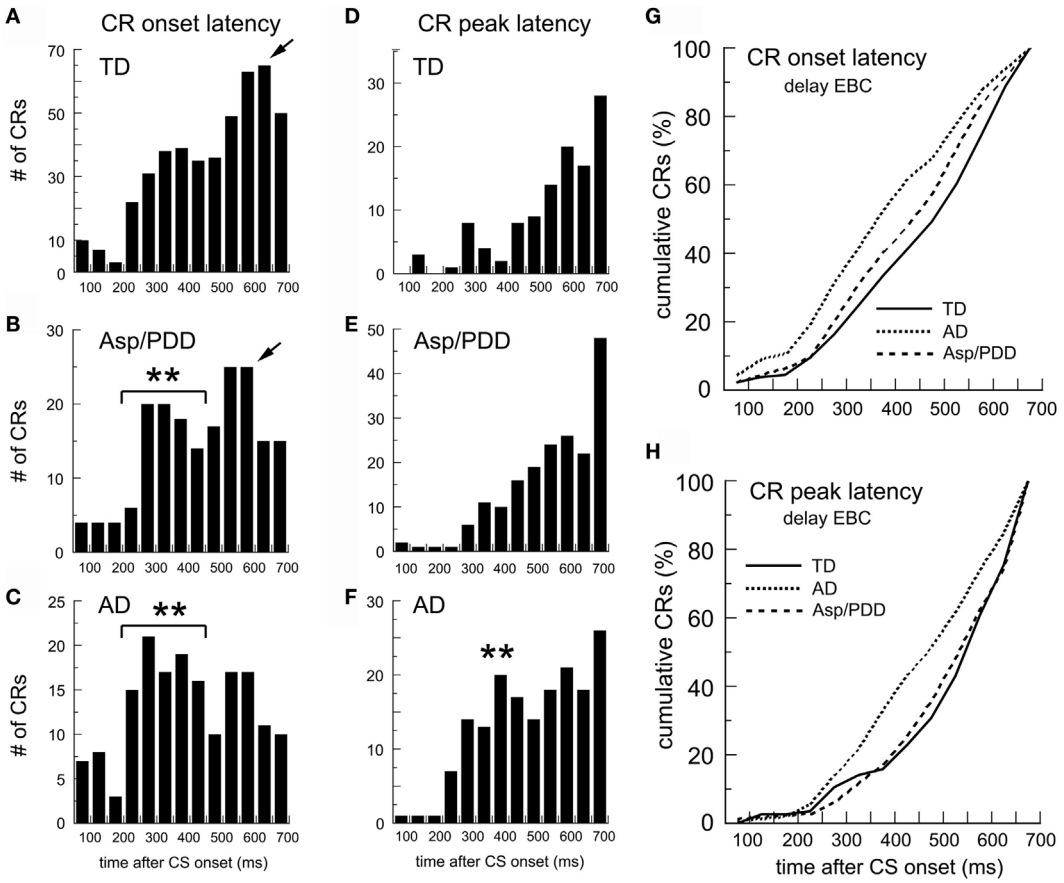

FIGURE 3 | CR timing during delay EBC. (A–C) Post-CS time histograms of CR onset. **(D–F)** CR peak latency histograms. **(G,H)** Cumulative probability plots of CR onset and CR peak. Bin width = 50 ms. Time 0 = CS onset. Arrows show mode onsets. Asterisks indicate early CRs.

and AD ($D = 0.21$, $p < 0.001$), but not between TD and Asp/PDD ($D = 0.06$, n.s.). Mood's median test also detected a highly significant difference between the CR peak latencies of TD and AD ($X^2 = 10.2$, $p < 0.01$), but not between TD and Asp/PDD ($X^2 = 1.4$, n.s.). Thus, there was a strong indication of a decrease in CR peak latency for AD (62 ms), but no indication of a change for Asp/PDD.

Table 1 presents the outcomes of the above comparisons. In sum, there was moderate indication that Asp/PDD, but not AD, was associated with a small increase in CR onset and peak latency during trace EBC. There was a strong indication that subjects with AD had significantly reduced CR onset and peak latencies during delay EBC. Reduced CR onset latencies during delay EBC were also observed in the Asp/PDD group, but to a smaller degree, and, unlike the AD group, the CR peak latency was not significantly different for Asp/PDD during delay EBC.

Table 1 | Magnitude and direction of CR performance changes for the AD and Asp/PDD groups.

	CR onset latency			CR peak latency		
	K–S	*t*-test	Median	K–S	*t*-test	Median
Trace						
AD	**Yes	No	No	*Yes	No	No
Asp/PDD	***Yes	**Yes ↑	No	**Yes	**Yes ↑	No
Delay						
AD	***Yes	***Yes ↓↓	***Yes ↓↓	***Yes	***Yes ↓↓	**Yes ↓↓
Asp/PDD	***Yes	*Yes ↓	No	No	No	No

Arrows indicate directionality of change (up arrow = increased latency; down arrow = decreased latency), relative to the TD group.
Color indicates the statistical strength of performance change (green = consistently positive; yellow = moderate; red = consistently negative).
**p < 0.05, **p < 0.01, ***p < 0.001, compared to TD.*

Figure 4A plots the average topography of the CRs on the first 30 trials of delay EBC. The most significant deviation from the monophasic waveform of the TD group (**Figure 4A**, green) was the presence of two peaks in the average CR of the AD group (**Figure 4A**, red), with the first peak at 350 ms (**Figure 4A**, arrow) and the second at 600 ms (**Figure 4A**, arrowhead on red trace). The average CR of the AD group also differed from Asp/PDD waveform, which also showed only a single peak at approximately 600 ms (**Figure 4A**, arrowheads). The two peaks in the average CR of the AD group was consistent with either a biphasic CR or the averaging of two types of CRs having early and late timing. The analysis was repeated by selecting the

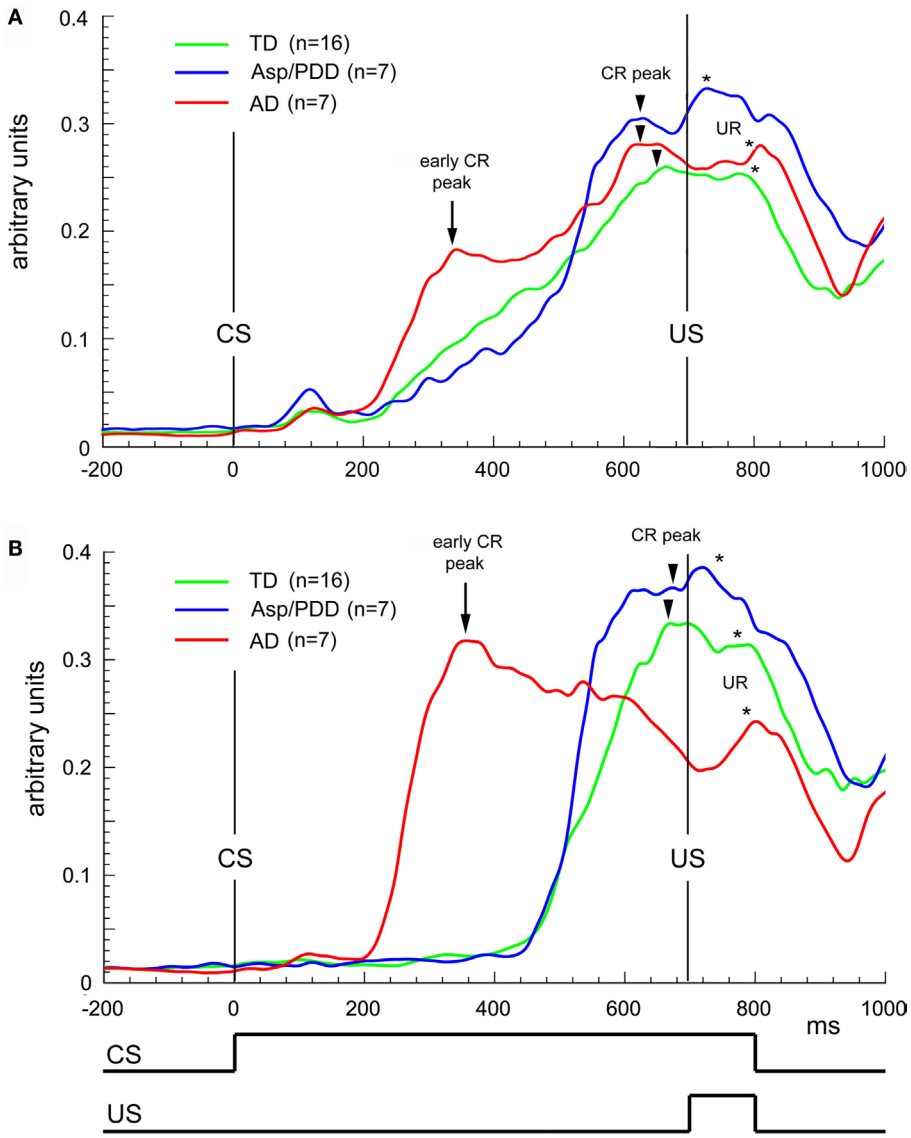

FIGURE 4 | **Waveform analysis during delay EBC**. **(A)** Average CRs during the first 30 trials of delay EBC for TD (green), Asp/PDD (blue) and AD (red) groups. **(B)** Average of the most prevalent type of CR for each group. Arrows indicate abnormally early CR peaks by the AD group. Arrowheads indicate CR peak close to US onset. Asterisks indicate unconditioned response peaks to the airpuff US. Curves show the mean of all subjects in each group.

CRs having modal onsets within each of the groups. For the AD group, this corresponded to CRs with onsets between 200 and 450 ms (**Figure 4B**, red) detected in the onset histograms (**Figure 3**), while, for the other two groups, this corresponded to CRs with onsets between 500 and 650 ms. The early CRs of AD subjects showed only one, abnormally early peak that occurred at 350 ms (**Figure 4B**, arrow), thereby accounting for the first of the two peaks in the average CR and indicating that they were not biphasic CRs but rather monophasic CRs that were inappropriately timed. Notably, those early CRs did not maintain peak amplitude throughout the CS–US interval, unlike the average CR of TD and Asp/PDD that peaked within 50 ms of the US (**Figures 4A,B**, arrowheads).

There was significant heterogeneity among the subjects with regard to CR performance. **Figure 5** shows plots of normalized values of CR onset latency vs. peak latency relative to the TD mean for every subject. It can be seen that the three groups overlapped on session 1, which was trace EBC (**Figure 5A**), and largely overlapped on session 2, which was also trace EBC (**Figure 5B**). Of note was that three of seven AD subjects during the second trace EBC session moved into the lower half of the TD distribution, while the Asp/PDD distribution did not shift. During session 3, which was delay EBC (**Figures 5C**), five of seven AD subjects separated further and fell below both the TD (**Figure 5C**, green lines) and Asp/PDD (**Figure 5C**, blue lines) means for both CR onset and peak latency, with two AD subjects

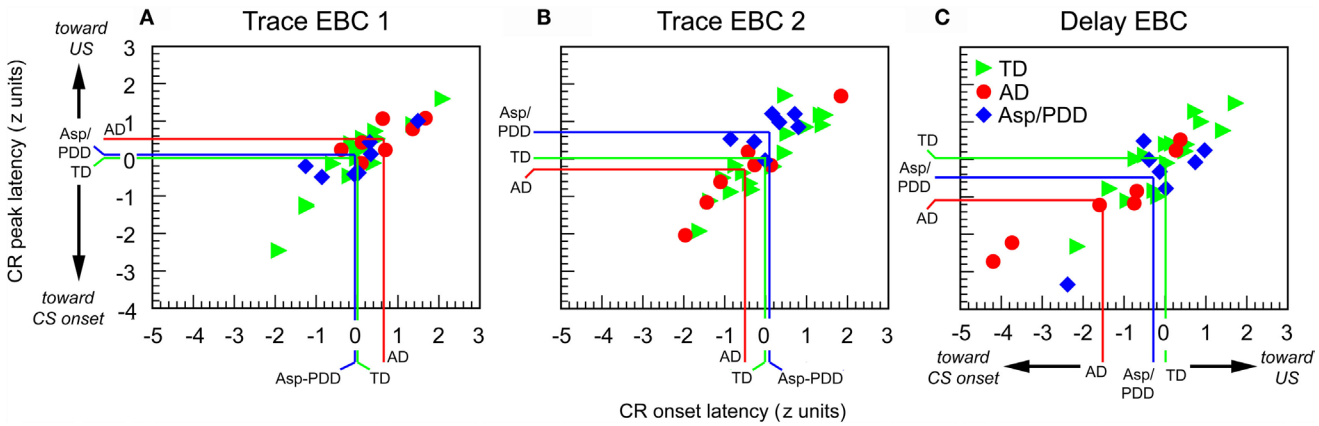

FIGURE 5 | Individual CR timing data during the first 30 trials of each EBC session. Data are normalized to the TD group mean and expressed as standard scores. Colored lines indicate means of TD (green), AD (red), and Asp/PDD (blue) groups.

far outside the TD range. During delay EBC, the mean deviations of CR onset and peak latency were 1.5 and 1.1 SDs from the TD mean, respectively (**Figure 5C**, red lines).

DISCUSSION

There are two reports in the literature that have described alterations in classical EBC in high-functioning subjects with ASD (1, 3). Both reports used a heterogeneous subject pool, either due to a wider age range than is standard in current ASD research (3) and/or due to the pooling of current diagnostic categories within the ASD spectrum. Here, we reexamined the data presented in Oristaglio et al. (1) that pooled children diagnosed with AD, Asp, and PDD-NOS into one group. By separating subjects with AD and analyzing two subgroups, this is the first description of differential effects of ASD diagnoses on CR performance in high-functioning children. A limitation of the present study is the small number of subjects within the groups. Thus, our preliminary findings warrant replication with larger populations of children along the ASD spectrum and at different ages. Advantages of EBC are the stereotypic nature of CR performance and the fact that EBC can be applied across a wide range of cognitive functioning in a standardized manner. Because EBC is a robust test of associative learning and motor timing that interrogates the functioning of the cerebral and hindbrain–cerebellar systems in trace and delay paradigms, respectively, it may have further utility for evaluating brain dysfunction in pediatric populations with ASD.

By disaggregating diagnostic groups, we determined that subjects with AD were largely responsible for the finding that ASD is related to shorter CR onset and peak latencies during delay EBC (1, 3). The sensitivity of CR performance during the early stages of delay EBC in the AD group was indicated by three statistical tests that evaluated changes in central tendency and distribution shape. The analysis provided the new finding that subjects with Asp/PDD, but not those with AD, showed a difference in motor timing during trace EBC in which both CR onset and peak latencies were delayed. The magnitude of that effect was much smaller than the reduced CR latency shown by AD subjects during delay EBC, but may have important consequences for understanding brain regions that may be differentially impacted in diagnostic subcategories of ASD. Because decreases in CR onset and peak latency during delay EBC have been associated with damage to the cerebellar cortex (12, 13), the EBC phenotype with AD is consistent with a potential cerebellar involvement in the CR performance change. Because Asp/PDD subjects showed slightly shorter CR onset latencies during delay EBC and no change in CR peak latency, this may be consistent with more subtle cerebellar involvement than in AD. Of particular relevance is the finding that there is significant heterogeneity among individuals with ASD in the loss of Purkinje cells in cerebellar cortex (14). Although the loss of Purkinje cells or other cell types in the cerebellum may not pertain to the majority of cases of ASD, changes in excitability and/or plasticity in cerebellar and pre-cerebellar neurons also may underlie CR performance changes in ASD (15), as confirmed in mouse models of tuberous sclerosis (16), Fragile X (4), and 15q11–13 duplication (6), all of which are conditions associated with ASD in humans. On the other hand, the slightly longer CR onsets that Asp/PDD subjects demonstrated during trace EBC potentially implicates an additional disruption in a telencephalic process that plays a larger role for specifying CR timing during trace EBC.

We observed that there is significant heterogeneity in CR performance within an ASD diagnostic group and that there is overlap between groups. For instance, two of seven subjects in the AD group showed normal CR timing during delay EBC, and one of seven subjects in the Asp/PDD group showed a change in CR performance as extreme as the most-affected AD subjects. The neurobiological causes of these effects remain to be elucidated, but future studies of brain morphology and neurochemistry may be helpful (17, 18).

The observation of heterogeneity in EBC performance at different points along the ASD spectrum is consistent with the contrast between high-functioning children with idiopathic ASD and individuals with Fragile X syndrome, a form of severe intellectual disability in which approximately 50% have comorbid symptoms of ASD. As two reports confirm (1, 3),

high-functioning individuals with ASD are not impaired in their ability to acquire CRs, but many have CR onset and peak latencies during delay EBC that are earlier than normal. In the case of Fragile X, affected individuals similarly show earlier CR peak latencies during delay EBC, but also a prominent reduction in CR acquisition in subjects older than 45 years (4, 5). Our preliminary indication that children with Asp/PDD show an increase in CR latency during trace EBC and a small decrease in CR latency during delay EBC, while children with AD show normal CR performance during trace EBC but a large decrease in both CR onset and peak latency during delay EBC helps further indicate that there is heterogeneity in CR performance changes across the ASD spectrum.

Understanding the genetic predispositions for the magnitude and direction of EBC performance changes across the ASD spectrum will be a promising direction for future clinical studies. A recent report (7) of CR performance during delay EBC to a light CS in mouse models of idiopathic and syndromic ASD indicated that monogenetic mutations can have differential effects on CR performance. For instance, a globally expressed truncation mutation in Shank3 decreased CR peak latency by approximately 30 ms, and a truncation mutation in MeCP2 increased CR peak latency equivalently. However, global Cntnap2 knockout, 15q(11–13) duplication, and Purkinje cell-specific knockout of tuberous sclerosis protein had no effect on CR timing despite being models of human ASD. It is noteworthy that the overall magnitude of the CR performance changes in our AD group during delay EBC was much larger than observed in any mouse model produced by monogenetic deletion or mutation, which may reflect the polygenetic nature of idiopathic autism in humans that affects brain development, connectivity, and synaptic physiology to various degrees across individuals (19). Moreover, understanding the contributions that alterations in sensory processing may play in affecting EBC performance across the ASD spectrum will be important, as there is significant heterogeneity among individuals in the directionality of changes in sensitivity that can differ across sensory modalities. It could be important to determine whether the delays in tone-evoked potentials and high-frequency oscillations in the superior temporal gyrus (20) observed in children with ASD having language impairment relate to delays in CR performance during trace EBC with an auditory CS, generally believed to require greater cerebral involvement than delay EBC, as well as whether any of the effects on CR performance reported here generalize to visual or tactile CSs.

As previously discussed (1), the abnormally short-latency CRs during delay EBC in the present cohort of high-functioning children with ASD replicate the major effect reported by Sears et al. (3) in a smaller group of individuals having ASD that spanned a larger-age range. Interestingly, in both Sears et al. (3) and Oristaglio et al. (1), the changes in CR timing with idiopathic ASD were not accompanied by an impaired ability to acquire CRs. Two differences between the results of our study and Sears et al. (3) were that the latter study observed enhanced CR acquisition in subjects with ASD relative to subjects with TD and overall greater asymptotic percent CRs than in our study. Those differences may be more apparent than real, however, for six reasons. First, Sears et al. (3) employed delay EBC exclusively, while our subjects were initially trained on trace EBC, a more difficult task that typically results in more modest learning performance in both human and animal studies. Second, Sears et al. (3) employed a 350 ms CS–US interval and an intensity of a tone CS that is about double in perceived loudness, both of which are well known to increase the rate of CR acquisition and the asymptotic percent CRs as compared to the 700 ms CS–US interval and 61-dB tone employed in our study (21). We specifically chose the 700 ms CS–US interval to produce a more difficult learning paradigm that would enhance the ability to detect differences between diagnostic groups and a softer tone CS to prevent distress in the children. Third, Sears et al. (3) measured CRs with corneo-retinal potentials in contrast to our use of an infrared detector. This significant difference in method may have contributed to Sears et al.'s (3) increased detection of small amplitude CRs supported by both associative and non-associative factors. Fourth, the faster CR acquisition in Sears et al. (3) may indicate a heightened ability to process short CS–US intervals in ASD that is not apparent when a 700 ms CS–US interval is employed. That possibility is the most interesting with regard to ASD neurobiology and can be tested explicitly in the future using different CS–US intervals in a within-subject design. Fifth, we observed considerable variability in the rate of CR acquisition for children in our ASD subgroups, with some performing as well or better than TD subjects while others performing below the performance of the TD group. This may potentially be accounted for by heterogeneity in sensory processing that does not segregate according to the clinical criteria which define the diagnostic subgroups. Sixth, the significant heterogeneity in the causes and symptoms of ASD and the fact that the ASD populations of Sears et al. (3) and Oristaglio et al. (1) were ascertained by different clinicians separated by 20 years, over which time inclusion/exclusion criteria and treatment have gradually shifted, could have contributed to the differences in CR acquisition, while greatly increasing the significance of the replicated effect on CR timing. Additional clarification will require studies of much larger ASD populations than has been performed to date.

In sum, our study provides an initial examination of the utility of trace and delay EBC for distinguishing ASD subgroups and suggests differential effects and heterogeneity of CR performance between and within subgroups. This provides a reference dataset for future studies of larger populations of children and non-human animals that may examine the genetic and neurobiological bases of the directionality and magnitude of CR performance differences with ASD and whether these differences generalize to other forms of sensory-motor timing.

AUTHOR CONTRIBUTIONS

JW and JO analyzed the data and wrote the paper.

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Eyeblink classical conditioning in alcoholism and fetal alcohol spectrum disorders

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Alcoholism is a debilitating disorder that can take a significant toll on health and professional and personal relationships. Excessive alcohol consumption can have a serious impact on both drinkers and developing fetuses, leading to long-term learning impairments. Decades of research in laboratory animals and humans have demonstrated the value of eyeblink classical conditioning (EBC) as a well-characterized model system to study the neural mechanisms underlying associative learning. Behavioral EBC studies in adults with alcohol use disorders and in children with fetal alcohol spectrum disorders report a clear learning deficit in these two patient populations, suggesting alcohol-related damage to the cerebellum and associated structures. Insight into the neural mechanisms underlying these learning impairments has largely stemmed from laboratory animal studies. In this mini-review, we present and discuss exemplary animal findings and data from patient and neuroimaging studies. An improved understanding of the neural mechanisms underlying learning deficits in EBC related to alcoholism and prenatal alcohol exposure has the potential to advance the diagnoses, treatment, and prevention of these and other pediatric and adult disorders.

Keywords: alcoholism, ethanol, cerebellum, fetal alcohol spectrum disorders, eyeblink classical conditioning, associative learning

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INTRODUCTION

Alcohol is one of the most widely abused substances in the world (1) and can have a major impact on health and professional and personal relationships. One reason for this negative societal impact is that excessive alcohol consumption often leads to long-term learning and memory impairments. In this mini-review, we will outline exemplary animal and human findings that guide our current understanding of how chronic alcohol exposure alters neural structure and function underlying a fundamental form of learning, eyeblink classical conditioning (EBC). Specifically, this mini-review will focus on alcohol use disorders (AUD) in adults and fetal alcohol spectrum disorders (FASD) in children.

One area of the brain that is targeted in AUD and FASD is the cerebellum (2, 3). Although excessive alcohol consumption affects many other brain regions (4–6), this mini-review will focus on the cerebellum due to its critical involvement in EBC (7) and the particular vulnerability of the

cerebellum to alcohol exposure (8, 9). This line of research has produced overwhelming evidence that the cerebellum and associated structures are critically important for EBC. Specifically, contributions from the cerebellar cortex, particularly in lateral lobule VI (10, 11), and cerebellar deep nuclei (12, 13) have been documented in both animals and humans. **Figure 1** depicts this well-documented circuitry.

Eyeblink classical conditioning involves the pairing of a neutral conditioned stimulus (CS; e.g., a tone) and an unconditioned stimulus (US; e.g., a corneal airpuff). The US is often a biologically salient stimulus sufficient to elicit an unconditioned response (UR; e.g., a blink). Following multiple CS–US pairings, an organism learns to produce a conditioned response (CR) in anticipation of the US presentation, suggesting that an association between the CS and US has been learned. EBC is a simple, yet elegant model of learning, which can already be assessed in humans by 5 months of age (14) and represents a foundation on which more complex learning is built (15, 16). Understanding the etiology of fundamental learning impairments that accompany alcohol-related disorders may have potential to foster new approaches to early diagnoses, intervention, and effective treatments and presents a model for studying effects of other pediatric and adult disorders as well as effects of other drugs or environmental contaminants.

LABORATORY ANIMAL WORK

Structural Alterations (Mature Cerebellum)

There is extensive laboratory animal evidence showing that chronic intake of alcohol is associated with neuroanatomical changes in the cerebellum (17). A common observation is shrinkage of the cerebellum. In the adult rat, these volumetric reductions may be due to death and atrophy of cells in the Purkinje, granular, and molecular layers of the cerebellar cortex (18–21). In addition to degenerative changes in cell bodies, morphological changes to dendrites and axons have also been reported (22–24). Combined treatments of thiamine deficiency and alcohol exposure have led to axon terminal degeneration in the deep cerebellar nuclei, the sole output region for the cerebellum (25). Fewer synapses between parallel fibers and Purkinje cells (26) and a significant decrease in the number of dendritic microtubules have been found in alcohol-fed adult rats (27). At the molecular and cellular level, γ-aminobutyric acid$_A$ (GABA$_A$) is altered by chronic alcohol consumption (28), whereas there is an overexpression of glutamate and a prolonged opening of mitochondrial permeability in the cerebellum following alcohol withdrawal (29).

Structural Alterations (Developing Cerebellum)

Cerebellar structural abnormalities also appear in the developing cerebellum as a result of excessive early alcohol exposure. This damaging effect appears to be sensitive to time of alcohol exposure as rats receiving alcohol on postnatal day 4 suffered up to 50% Purkinje cell loss, whereas later exposure (postnatal days 8/9) resulted in less severe (15%) cell loss (30, 31). Alcohol-related damage in granule cells has also been investigated and cell vulnerability again appears to be greatest early in development (postnatal days 4/5) (32, 33). The structural integrity of the cerebellar deep nuclei, a region believed to be crucially important for EBC memory formation and storage (7), has been shown to be susceptible to chronic alcohol consumption. Binge-like and moderate

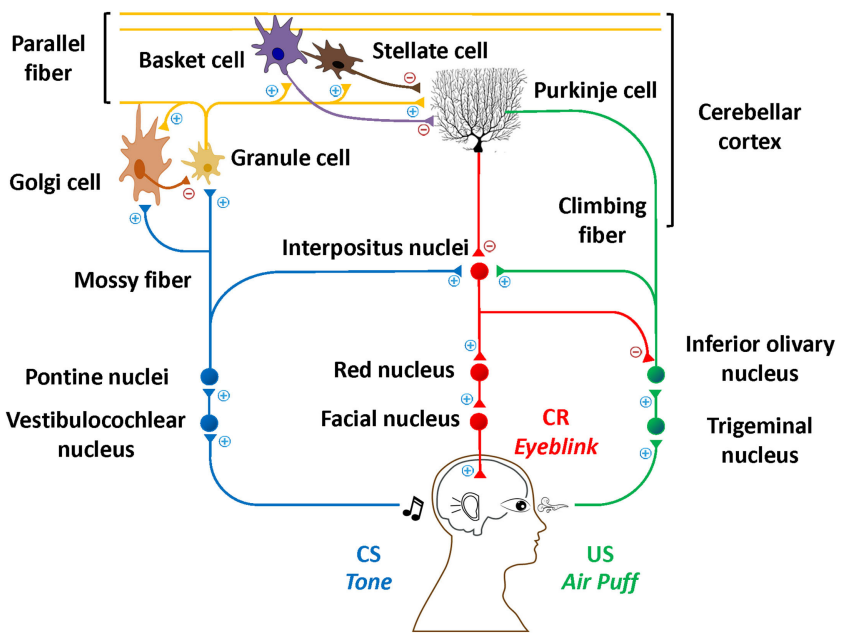

FIGURE 1 | Essential neural circuitry of eyeblink conditioning. Blue lines indicate the conditioned stimulus pathway. Green lines indicate the unconditioned stimulus pathway. Red lines indicate the conditioned response pathway. Excitatory and inhibitory synapses are represented by + and −, respectively.

neonatal exposure to alcohol was sufficient to produce behavioral deficits in EBC associated with significant deep nuclear cell loss in adult rats (34, 35). During development, even a single exposure to alcohol introduced subcutaneously was sufficient to promote cellular apopotosis in the deep cerebellar nuclei (36).

Functional Differences (Mature Cerebellum)

Abnormal cerebellar functioning is another consequence of chronic alcohol exposure. Very little attention has been given to the chronic effects of alcohol on the cerebellum in adult laboratory animals. To the best of our knowledge, only one study to date has examined these effects. In mature mice, chronic alcohol consumption resulted in a decrease in simple and complex spike firing and an increase in complex spike duration and pause in Purkinje cells but no differences were detected in Golgi cell firing patterns (37).

Functional Differences (Developing Cerebellum)

Most of our current knowledge on the functional consequences of chronic alcohol exposure stems from work on the developing cerebellum. Following alcohol exposure during pregnancy, *in vitro* experiments using a long-term depression (LTD) induction protocol showed parallel fiber long-term potentiation (LTP) in cerebellar slices in alcohol-exposed juvenile mice but LTD in control mice (38). Furthermore, *in vivo* experiments showed that simple spike firing rates in Purkinje cells increased and showed faster oscillations of local field potentials in exposed mice relative to controls (38). These exposed mice also exhibited impaired EBC, further supporting the hypothesis that cerebellar LTD in Purkinje cells is crucial for the timing of eyeblink CRs (39). Interestingly, other *in vitro* electrophysiology experiments showed that alcohol exposure led to relatively greater inhibitory inputs to the Purkinje cells in the vermis (40). In the cerebellar deep nuclei, activity in the interpositus nucleus of the cerebellum was diminished and did not develop as rapidly in neonatal alcohol-exposed rats relative to controls during EBC (41, 42).

Learning Deficits

Since the cerebellum is vulnerable to chronic alcohol exposure and this structure plays a critical role in EBC, prolonged alcohol use is likely to result in learning deficits. Surprisingly, to date, there are no laboratory animal eyeblink conditioning studies investigating the role of chronic alcohol consumption in adulthood.

By contrast, there have been several animal studies on effects of pre- and neonatal exposure. Neonatal rats exposed to alcohol during the equivalent of the human third trimester showed learning deficits in standard delay EBC (43) as well as more complex EBC protocols, including trace conditioning, discrimination, and reversal learning (44, 45). The effects of alcohol on EBC also appear to be dose dependent, with higher dosages producing greater impairments (45, 46). Binge-like and even moderate exposure to alcohol during development produces EBC deficits that persist into adulthood, suggesting long-lasting permanent cerebellar damage (35, 47). This evidence is consistent with studies that report a significant correlation between learning and the number of deep cerebellar nuclear cells in alcohol-exposed rats (34). Finally, interventions to ameliorate neonatal alcohol-related learning deficits have been met with mixed results. MK-801 administration, choline supplementation, and a combination of exercise and environmental enrichment mitigate behavioral EBC deficits, suggesting neuroprotective or other ameliorative effects (48–50), whereas vitamin E did not reduce alcohol-related EBC deficits (51).

HUMAN WORK

Structural Alterations (Mature Cerebellum)

Consistent with laboratory animal findings, human data also indicate that chronic alcohol consumption has harmful effects on the structural integrity of the adult cerebellum (4, 52). Structural MRI has revealed gray matter reductions in the cerebellar hemispheres and vermis in AUDs (53). Furthermore, cerebellar gray matter volume loss was correlated with poor neuropsychological performance and early age of first drinking (54). Diffusion tensor imaging (DTI) showed that recovered AUDs had diminished white matter fibers relative to healthy controls, suggesting that impaired connectivity may partially mediate some of these behavioral deficits (55). Human histological studies report significant Purkinje cell loss in the cerebellar hemispheres and vermis as a result of years of alcohol abuse (9, 56, 57).

Structural Alterations (Developing Cerebellum)

As indicated above, animal models predict that alcohol exposure damages the developing cerebellum. These findings are also consistent with human studies: autopsy reports of children prenatally exposed to large quantities of alcohol describe malformations in the cerebellum characterized by reduced size and disorganization (58). In addition, cerebellar dysgenesis was reported in 10 of 16 FAS autopsies (59). Modern neuroimaging data agree with these observations, as exposed children had proportionately greater reductions in cerebellar cranial vault and volume (60, 61), including a 15% reduction in cerebellar volume in children with FAS (8). Specifically, significantly smaller cerebellar hemispheres and vermis were found in exposed relative to healthy children (62, 63). Differences in white matter integrity [lower fractional anisotropy (FA) and greater perpendicular diffusivity] between alcohol-exposed and non-exposed children have been identified in the middle cerebellar peduncles, fibers shown to be important in animal models of EBC (64, 65). Children with FAS also showed lower FA bilaterally in the superior peduncles. Finally, using *in vivo* (1) H magnetic resonance spectroscopy (MRS) to examine neurochemical differences in the cerebellar deep nuclei, Du Plessis et al. (66) found that prenatal alcohol exposure was associated with lower levels *N*-Acetylaspartate (NAA) and glycerophosphocholine + phosphocholine (Cho) and higher levels of glutamate plus glutamine (Glx).

Functional Differences (Mature and Developing Cerebellum)

Consistent with these structural findings, evidence from functional magnetic resonance imaging (fMRI) studies suggests fMRI brain activations are also affected by alcoholism. In a finger tapping task, AUD subjects tended to exhibit more extensive and bilateral cerebellar activation than healthy controls (67). Greater right superior cerebellar activity during a Sternberg working memory task was assessed in AUD subjects (68). In an auditory language task, AUD subjects showed greater fMRI activations in the cerebellar vermis, despite comparable behavioral performance to healthy controls (69). Children diagnosed with fetal alcohol syndrome (FAS) or partial FAS (PFAS) showed greater cerebellar activation in a working memory n-back task relative to healthy children (70). Rhythmic tapping elicited greater activation in children with FASD in crus I and vermis IV–V (71). This pattern of greater activation by adults and children may represent compensatory mechanisms during each task.

Learning Deficits

Similar to laboratory animals, humans also show alcohol-related deficits in EBC. Impaired standard delay eyeblink conditioning (CS and US co-terminate) was seen in amnesic Korsakoff patients and recovered, uncomplicated AUDs (72). These findings were extended to more complex conditioning protocols. During temporal discrimination, in which two distinct CSs with two different interstimulus intervals (ISI) were presented, AUDs' peak CR latency at the long ISI was significantly shorter relative to healthy controls, demonstrating a deficit in adaptive CR timing (73). Trace conditioning is a procedure that incorporates a stimulus free period between offset of the CS and onset of the US. Naive AUDs showed learning deficits in trace conditioning, whereas AUDs previously trained in delay conditioning showed comparable trace conditioning to naive control subjects (74). AUDs who were successful at learning a delay discrimination protocol (i.e., learn that one CS predicts the US, whereas another CS predicts its absence) were impaired when the contingencies were reversed, suggesting an inability to learn new adaptive associations (75).

Similar to adults, children with FASD demonstrate remarkably consistent conditioning deficits. In a cross-sectional study comparing children with FASD, attention deficit hyperactive disorder (ADHD), dyslexia, and healthy controls, the children with FASD and dyslexia showed conditioning impairments relative to the healthy children and different patterns than those seen in children with ADHD (76). In the first prospective

TABLE 1 | Effects of alcohol on cerebellar structure, function, and eyeblink conditioning reported in the literature.

	Animals		Humans	
	Reference	Comments	Reference	Comments
Structural alterations	(32)	Purkinje and granule cell loss (D)	(9)	Purkinje cell volume loss (M)
	(36)	Purkinje and deep cerebellar nuclear cell loss (D)	(8)	Cerebellar volume loss (D)
	(30)	Purkinje cell loss (lobules I–V, IX, and X) (D)	(63)	Hypoplasia of cerebellar vermis (D)
	(34, 35)	Deep cerebellar nuclear cell loss (D)	(54)	Cerebellar gray matter loss correlated with neuropsych. tests (M)
	(33)	Purkinje and granule cell loss (postnatal days 4–5) (D)	(55)	Diminished white matter fiber (M)
	(21)	Purkinje and granule cell loss (M)	(59)	Cerebellar dysgenesis in 10 of 16 FAS autopsies (D)
	(27)	Dendritic microtubules loss (M)	(58)	Cerebellar reduction and disorganization (D)
	(24)	Longer terminal dendritic segments in Purkinje cells (M)	(66)	Differences in cerebellar neurochemisiry (D)
	(25)	Deep cerebellar nuclear axon terminal degeneration (M)	(65)	Cerebellar peduncles damage (D)
	(18)	Granule cell loss (M)	(60, 61)	Reductions in cerebellar cranial vault and volume (D)
	(19, 22)	Longer and reduced Purkirje dendritic spines (M)	(57)	Cell loss in cerebellar vermis (M)
	(23)	Increased climbing fibers (M)	(62)	Cerebellar vermis volume reduction (D)
	(20)	Purkinje and granule cell loss (M)	(64)	Cerebellar peduncles damage (D)
	(26)	Fewer synapses between parallel fibers and Purkinje cells (M)	(53)	Cerebellar vermis gray matter deficits (M)
	(31)	Purkinje cell loss (postnatal days 4–5) (D)	(56)	Reduced Purkinje cell density in the vermis (M)
Functional differences	(41)	No single-unit activity changes in cerebellar deep nuclei (D)	(69)	Greater fMRI activity in cerebellar vermis (M)
	(40)	Greater inhibitory inputs to Purkinje cells (D)	(68)	Greater fMRI responses in lobule VI (M)
	(42)	Slower increases in deep nuclear activity (D)	(70)	Greater cerebellar fMRI activation (D)
	(37)	Purkinje cell firing differences (M)	(71)	Greater crus I and vermis IV–V activation (D)
	(38)	Purkinje cell firing differences (D)	(67)	More extensive cerebellar fMRI activation (M)
Learning deficits	(44)	Impaired EBC discrimination learning (D)	(76)	Impaired delay EBC (D)
	(34, 35, 47)	Impaired delay EBC (D)	(75)	Impaired EBC discrimination and reversal learning (M)
	(45)	Impaired trace EBC (D)	(77, 78)	Impaired delay and trace EBC (D)
	(43)	Impaired delay EBC (D)	(74)	Impaired trace EBC (M)
			(72, 73)	Impaired delay and temporal EBC discrimination (M)

A summary of animal and human work investigating how excessive alcohol consumption affects the cerebellum and eyeblink conditioning. M and D indicate effects on the mature and developing cerebellum, respectively.

longitudinal study on EBC in children with FASD, Jacobson et al. (77) extended these findings by presenting additional trials (up to 150 trials) to 5-year-old children diagnosed with FAS, PFAS, heavily exposed non-syndromal (HE) children, and controls. Despite the additional training opportunity, none of the children with FAS met criterion for conditioning, whereas 75% of the controls did (77). In another cohort of school-aged children, 66.7% of the children with FAS failed to meet criterion on the delay task, and only 16.7% of the FAS and 21.4% of HE group met criterion for trace conditioning in comparison to 66.7% of healthy controls (78). Odds ratio data from a logistic regression analysis showed that the children with FAS were 7.7 times more likely to fail to meet criterion on the delay task compared with controls and 10.0 times more likely on the trace conditioning task. Similarly, the HE group was 5.1 times more likely to fail to meet criterion on delay and 7.3 times more likely on trace. In both the 5-year and school-age studies, IQ did not differentiate the children who reached criterion on delay and trace EBC from those who failed, indicating that it could not be a mediator of the effect of fetal alcohol exposure on performance on either EBC task; nor was ADHD responsible for the observed alcohol-related pattern of EBC impairment seen in the two cohorts. Collectively, these findings strongly support the view that prenatal alcohol exposure has deleterious effects on children's ability to demonstrate successful EBC and thus has the potential to serve as a biobehavioral marker of prenatal alcohol impairment as well as a useful tool to assess the efficacy of an intervention (79).

DISCUSSION

The damaging effects of alcoholism on the cerebellum and EBC have been well-documented in animal and human investigations. This mini-review summarizes some exemplary laboratory animal and human studies (see **Table 1**). Chronic, excessive alcohol consumption leads to neuroanatomical alterations in the adult and/or fetal cerebellum, including neuronal loss and white matter degradation. Alcohol exposure also triggers abnormal cerebellar activity as shown through electrophysiology and neuroimaging methodologies. The combination of these effects likely underlies the conditioning deficits seen by these two populations.

One limitation in this field of study is that alcohol affects multiple regions of the brain outside the cerebellum. Affected and connected areas may exert influences on cerebellar structures, making results difficult to interpret. Future work should consider the cerebellum as part of a larger network. This fundamental associative learning task is clinically relevant because it represents a foundation on which more complex learning is built. Studies of environmental exposures, such as alcohol, on EBC have the potential to provide new information about the EBC neural circuitry and behavioral performance and to elucidate vulnerable neural structures that are uniquely recruited during basic learning processes. A comparison of EBC and neuroimaging findings between adults with AUD and children with FASD to determine common neuroanatomical targets of alcohol abuse is an important goal. Moreover, EBC has the potential to identify impairment related to different exposures and in different pediatric and adult disorders, such as ADHD, schizophrenia, FASD, and AUD. This work could lead to assessment of degree of behavioral and cerebellar impairment in AUD and aid in early identification of fetal alcohol-affected children as well as assessment of efficacy of new interventions and treatments. Future interventions could involve the use of neuromodulatory tools, such as transcranial magnetic stimulation and transcranial direct current stimulation, as a way to alter brain activation in an effort to improve learning in AUD and FASD individuals. Finally, this learning model could also be used to identify at-risk individuals, thereby leading to effective prevention strategies.

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