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Biobehavioral Markers of Adverse Effect in Fetal Alcohol Spectrum Disorders

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Abstract

Identification of children with fetal alcohol spectrum disorders (FASD) is difficult because information regarding prenatal exposure is often lacking, a large proportion of affected children do not exhibit facial anomalies, and no distinctive behavioral phenotype has been identified. Castellanos and Tannock have advocated going beyond descriptive symptom-based approaches to diagnosis to identify biomarkers derived from cognitive neuroscience. Classical eyeblink conditioning and magnitude comparison are particularly promising biobehavioral markers of FASD—eyeblink conditioning because a deficit in this elemental form of learning characterizes a very large proportion of alcohol-exposed children; magnitude comparison because it is a domain of higher order cognitive function that is among the most sensitive to fetal alcohol exposure. Because the neural circuitry mediating both these biobehavioral markers is well understood, they have considerable potential for advancing understanding of the pathophysiology of FASD, which can contribute to development of treatments targeted to the specific deficits that characterize this disorder.

Keywords

Fetal alcohol syndrome; Eyeblink conditioning; Arithmetic; Fetal alcohol spectrum disorders; Biomarkers; Behavioral phenotype; Prenatal alcohol exposure

Despite four decades of highly productive research since the first descriptions of fetal alcohol syndrome (FAS; Lemoine et al. 1968; Jones and Smith 1973), research on and treatment of fetal alcohol spectrum disorders (FASD) continue to be hampered by a lack of specificity in behavioral diagnostic criteria and limited understanding of the neural substrates that mediate the observed cognitive deficits. FAS, the most severe form of FASD, is characterized by a distinctive set of facial anomalies (short palpebral fissures, flat midface, thin upper lip, flat or smooth philtrum), accompanied by microcephaly and pre- or postnatal growth retardation (Jones and Smith 1973; Hoyme et al. 2005). Partial FAS (PFAS) is diagnosed when there is a history of heavy maternal drinking during pregnancy, two of the three alcohol-related facial anomalies, and at least one of the following: microcephaly, growth retardation, or cognitive and/or behavioral dysfunction. Alcohol-related neurodevelopmental disorder (ARND), the most prevalent but most difficult to identify of the disorders, is applied to individuals with confirmed prenatal alcohol exposure who lack the craniofacial anomalies of FAS but exhibit measurable, albeit often subtler neurobehavioral deficits (Stratton et al. 1996).

FASD is associated with a broad range of neurobehavioral deficits (see Mattson et al. 2011), including lower IQ (Streissguth et al. 1990; Jacobson et al. 2004), poorer attention and executive function (Coles et al. 1997; Kodituwakku et al. 1995; Carmichael-Olson et al. 1998; Mattson et al. 1999; Burden et al. 2005a), poorer verbal learning and memory (Mattson et al. 1996; Kaemingk et al. 2003; Coles et al. 2010), and slower cognitive processing speed (Streissguth et al. 1990; Jacobson et al. 1993, 1994; Coles et al. 2002; Burden et al. 2005b). Diagnosis of fetal alcohol-related disorders is difficult because information regarding prenatal exposure is often lacking, a large proportion of affected children do not exhibit the distinctive facial anomalies, and no distinctive behavioral phenotype has been identified. Although objective criteria have been developed to diagnose the facial anomalies and growth retardation associated with FAS and PFAS (e.g., Astley and Clarren 2001; Hoyme et al. 2005), cognitive and behavioral deficits have been reported in so many diverse domains that the 1996 Institute of Medicine (IOM) report concluded that any “evidence of...behavior or cognitive abnormalities...inconsistent with developmental level” must be deemed sufficient for a diagnosis of ARND (Stratton et al. 1996). In light of the broad range of neurobehavioral deficits reported in individuals with prenatal alcohol exposure, the IOM panel questioned the feasibility of distinguishing the neurobehavioral problems in FASD from those in other “individuals with complex intellectual deficits,” such as attention deficit hyperactivity disorder (ADHD).

Advances in neuroscience now make it possible to move beyond descriptive, symptom-based approaches to identify neurobehavioral endpoints specifically related to the etiology of FASD that could contribute to a better understanding of the underlying impairment resulting from prenatal alcohol exposure and lead to improved differential diagnosis and development of innovative treatments. Studies applying magnetic resonance imaging (MRI) to the study of FASD have found structural abnormalities in the cerebellum, parietal lobes, corpus callosum, and caudate nucleus (Archibald et al. 2001; Sowell et al. 2001b, 2002, 2008a; Lebel et al. 2011), particularly in the most severely affected individuals, and eight recent studies have documented fetal alcohol-related deficits in white matter integrity using diffusion tensor imaging (DTI) (Fryer et al. 2009; Lebel et al. 2008, 2010; Li et al. 2009; Ma et al. 2005; Sowell et al. 2008b; Wozniak and Muetzel 2011; Wozniak et al. 2006,

2009). However, few human studies have directly linked specific alcohol-related behavioral deficits with structural abnormalities (e.g., Sowell et al. 2001a, 2008a) or examined neural circuitry in relation to alcohol-related neurobehavioral impairment using functional MRI (fMRI).

Because most standard neuropsychological tests are complex and multifaceted, they provide little information about the specific aspects of CNS function that may be adversely affected in FASD. Phenotypically similar behavioral deficits seen in different disorders, such as FASD and ADHD, are hard to distinguish despite different etiologies, although several studies have made significant progress in this area (Coles et al. 1997; Vaurio et al. 2008; Burden et al. 2010; Kooistra et al. 2010; Jacobson et al. 2011a). To address this problem, Castellanos and Tannock (2002) have advocated going beyond descriptive symptom-based approaches to diagnosis to identify specific biomarkers derived from cognitive neuroscience that are “closer to the site of the primary causal agent” than to the manifest behavioral phenotype (p. 619). In studies focusing on genetic susceptibility to psychiatric disease, these biomarkers are termed endophenotypes (Gottesman and Gould 2003). With regard to FASD, the term *biomarker* can be used to refer to a biological marker of alcohol exposure or a biological endpoint that indicates pathology, like microRNA or protein changes related to pathology. We will use the term *biobehavioral marker* to refer to a behavioral endpoint linked to FASD whose neural substrates have been identified and can be examined directly. Identification of *biobehavioral* markers can enable research on FASD to go beyond the search for core deficits to investigate deficits linked to a specific brain region, circuit, chemical imbalance, or pattern of neural activation. In FASD, biobehavioral markers would consist of endpoints whose neural pathways or processes are altered by prenatal alcohol exposure and which mediate the observed neurobehavioral deficits.

Biobehavioral markers of effect are to be distinguished from *biomarkers of exposure*, such as the fatty acid ethyl ester (FAEE) metabolites of alcohol that are trapped in meconium during the second and third trimesters of pregnancy, which can provide a biological indicator of fetal alcohol exposure (Bearer et al. 1999, 2003). Biomarkers of exposure are invaluable for corroborating maternal report of drinking during pregnancy, but they provide no indication regarding the degree of impairment that has resulted from this exposure. Thus, in addition to biomarkers of exposure, biobehavioral markers of effect are needed to identify who among the exposed individuals have been adversely affected. Biobehavioral markers also have the potential to improve diagnosis by advancing our understanding of the neural substrates that mediate the effects of fetal alcohol exposure. Improved understanding of the pathophysiology of FASD can, in turn, contribute to the development of treatments that are better targeted to the specific deficits that characterize this disorder.

This paper will focus on two biobehavioral markers of fetal alcohol effect: eyeblink classical conditioning and magnitude comparison. Eyeblink conditioning (EBC) appears to be a particularly promising biobehavioral marker of FASD because a deficit in this elemental form of learning appears to characterize a very large proportion of alcohol-exposed children (Coffin et al. 2005; Jacobson et al. 2008, 2011b). Because the neural substrates of this form of conditioning have been exceptionally well documented in laboratory animals (Christian and Thompson 2003), research on this endpoint provides an excellent opportunity to investigate neural processes that mediate the adverse effects of fetal alcohol exposure. With regard to number processing, arithmetic is a domain of higher order cognitive function that is among the most sensitive to alcohol exposure in utero (Streissguth et al. 1991, 1994; Goldschmidt et al. 1996). Moreover, alcohol-related deficits in exact calculation appear to be mediated by a specific deficit in magnitude comparison, the fundamental ability to represent and manipulate quantity (Jacobson et al. 2011a). fMRI studies by Dehaene and others have identified a fronto-parietal circuit that mediates arithmetic calculation, providing

the basis for investigating the neuropathology that mediates the alcohol-related deficit in number processing. It is noteworthy that alcohol-related deficits in both EBC and arithmetic persist after control for IQ (Jacobson et al. 2008, 2011a, b; Carmichael-Olson et al. 1998; Goldschmidt et al. 1996), supporting the inference that they are specific features of FASD over and above the diminished overall intellectual ability associated with this disorder.

Eyeblink Conditioning

Classical eyeblink conditioning is a culturally neutral, nonverbal form of associative learning, in which the onset of a conditioned stimulus (CS), usually a pure tone, precedes an unconditioned stimulus (US), usually a mild air puff to the eye, which elicits a reflexive eyeblink unconditioned response (UR). Initially, the CS evokes no response but with repeated pairings of the tone and air puff, the tone CS comes to elicit an eyeblink response on a large percentage of trials. This eyeblink conditioned response (CR) represents the learned association between tone and air puff. The cerebellar cortex appears to regulate precise timing (latency and duration) of the CR, so that the CR occurs in anticipation of the air puff, thereby protecting the eye from the air puff.

EBC offers several advantages for the study of developmental neurobehavioral disorders (Stanton and Freeman 1994). The operational simplicity and minimal sensory, motor, and motivational demands of the procedure make it applicable with little or no modification across a range of animal species—rodents, rabbits, monkeys, sheep, humans— and across the life-span, beginning in early infancy (Stanton et al. 2010). As a behavioral procedure it has the advantage of offering parametric control of sensory, motivational, and associative variables; as well as a range of task variants that probe multiple brain “memory systems” (Stanton 2000). For example, two useful task variants are delay vs. trace conditioning. In delay conditioning, the tone CS precedes, overlaps, and co-terminates with the onset of the air puff, whereas in trace conditioning, there is a brief stimulus-free “trace interval” between the offset of the tone and the onset of the air puff (Fig. 1).

The neural substrates involved in eyeblink conditioning are well understood and have been documented in considerable detail in both humans and animal models (Woodruff-Pak and Steinmetz 2000a, b; Woodruff-Pak and Disterhoft 2008; Christian and Thompson 2003). Short delay conditioning (optimally with an interstimulus interval of 250–500 ms) depends on the cerebellum, whereas long delay and trace conditioning engages forebrain structures, such as the hippocampus and prefrontal cortex, in interactions with the cerebellum (Stanton 2000; Woodruff-Pak and Steinmetz 2000a, b; Woodruff-Pak and Disterhoft 2008). A well-defined brain stem-cerebellar circuit is both necessary and sufficient for delay conditioning (McCormick and Thompson 1984; Thompson 1986; Lavond et al. 1993; Logan and Grafton 1995; Kim and Thompson 1997; Thompson 2005). In delay conditioning, neural activity representing the tone CS is projected via the colliculus to discrete portions of the pontine nuclei, which convey this information to the cerebellum via mossy fibers in the middle cerebellar peduncle (Fig. 2). Neural activity representing the air puff US is projected via the inferior olive to the cerebellum via climbing fiber projections in the inferior peduncle. Both pontine and olivary inputs reach Purkinje cells in cerebellar cortex and send collateral inputs directly to the cerebellar deep nuclei. Neural plasticity in cerebellar cortex and deep nuclei produced by appropriately timed activation of climbing- and mossy-fiber inputs underlies short-delay conditioning (Thompson 1986, 2005; Krupa et al. 1993; Kim and Thompson 1997; Ohyama et al. 2003). The essential efferent CR pathway consists of fibers that project from the deep nuclei via the superior cerebellar peduncle to the red nucleus. The CR-related neural activity is then projected to the motor neurons that generate eyeblink CRs.

Reversible lesion studies using cold probe techniques and muscimol, a GABA_A agonist in the cerebellar interpositus nucleus abolished eyeblink CRs in previously trained animals and prevented acquisition of new CRs in naïve animals, whereas inactivation of the red nucleus prevented expression but not acquisition of CRs (Krupa et al. 1993; Clark and Lavond 1993; Clark et al. 1992) providing the strongest evidence to date that the cerebellar deep nuclei are critically involved in establishing the eyeblink CR (Steinmetz 2000; Thompson 2005). Two regions within the cerebellar cortex have also been implicated in EBC: cortical lesions restricted to the lobule HVI severely decrease the rate of conditioning and amplitude of the CR (Lavond and Steinmetz 1989), whereas lesions of portions of the anterior lobe disrupt normal timing of CRs (Perrett et al. 1993). The relative contributions of the deep nuclei and cerebellar cortex have also been examined using single-event fMRI in the conscious rabbit to visualize the entire cerebellum simultaneously during unilateral EBC training (Miller et al. 2003). Examination of the blood oxygenation level-dependent (BOLD) response indicated bilateral learning-related increases in the deep nuclei and deactivation in lobule HVI of the cerebellar cortex early in EBC training and continued bilateral BOLD response in the cortex but predominantly ipsilateral response in the deep nuclei later in training, as the learned response became increasingly refined. Cerebellar activity during EBC described in the rabbit model has been confirmed in young adult humans by positron-emission tomography (Logan and Grafton 1995) and fMRI (Cheng et al. 2008; Molchan et al. 1994; Schreurs et al. 1997).

The role of the hippocampus in trace conditioning has also been studied extensively in both humans and animal models. Hippocampal lesions have little or no effect on delay conditioning but severely disrupt acquisition of trace conditioning (Clark and Squire 1998; Port et al. 1986; Solomon et al. 1986; Moyer et al. 1990; Kim et al. 1995; McGlinchey-Berroth et al. 1997; Weiss et al. 1999; Ivkovich and Stanton 2001). Trace conditioning engages hippocampal plasticity in unit activity (Moyer et al. 1996; Weiss et al. 1996; McEchron and Disterhoft 1997; Power et al. 1997), induces changes in PKC expression (Van der Zee et al. 1997), induces increases in the number of multiple-synapse boutons (Geinisman et al. 2001), and increases survival of newly generated granule cells in adult rats (Gould et al. 1999; Shors et al. 2001).

Eyeblink conditioning emerges gradually over the course of development in both rodents (Stanton and Freeman 2000; Ivkovich et al. 2000; Ivkovich and Stanton 2001) and humans (Stanton et al. 2010). By 5 months post-term, normal human infants reach the same terminal level of conditioning as adults in the short delay procedure (Herbert et al. 2003). Whereas college age students show no trace-delay conditioning differences across a wide range of interstimulus intervals (Ross and Ross 1971), trace conditioning is more difficult for normal, middle class children (Werden and Ross 1972) and infants (Herbert et al. 2003) than for adults.

Eyeblink Conditioning in FASD

Heavy exposure to ethanol during the equivalent of the third trimester of pregnancy in humans disrupts EBC in weanling and adult rats, a deficit that is mediated by a dose-dependent cell loss and altered neural activity in the deep cerebellar nuclei (Green et al. 2002a, b). Binge exposure during this period in rodents is also associated with loss of Purkinje and granule cells in the cerebellum (Dunty et al. 2001; Hamre and West 1993) even after only 2 days of exposure (Thomas et al. 1998). Dikranian et al. (2005) have demonstrated that heavy ethanol exposure in rats and mice on even a single occasion during synaptogenesis triggers acute apoptotic neurodegeneration of Purkinje cells and other neurons in the deep cerebellar nuclei, cerebellar cortex, and two brainstem nuclei, the pontine nuclei and inferior olive, all elements of the EBC circuitry. Coffin et al. (2005) found poorer delay EBC in a sample of school-aged, alcohol-exposed children and in a

group of children with dyslexia, a disorder in which the cerebellum is believed to play an important role (Ivry et al. 2001). The alcohol-exposed children in the Coffin et al. study also had notable deficits in reading achievement, a problem previously linked to FASD (Streissguth et al. 1994; Goldschmidt et al. 1996; Molteno et al. in press). The absence of a conditioning deficit in a comparison group of children with attention deficit hyperactivity disorder (ADHD) in that study suggests that delay EBC may have a high diagnostic sensitivity for discriminating between individuals with different disorders who may exhibit similar behavioral problems.

The Cape Town Longitudinal Cohort Study

The incidence of FAS in the Cape Coloured (mixed ancestry) population in the Western Cape Province of South Africa has been estimated to be 18–141 times greater than in the United States (May et al. 2000). This population, composed mainly of descendants of white European settlers, Malaysian slaves, Khoi-San aboriginals, and black Africans, has historically comprised the large majority of workers in the wine-producing region of the Western Cape. The high prevalence of FAS in this community is a consequence of very heavy maternal drinking during pregnancy (Croxford and Viljoen 1999), which is due to poor psychosocial circumstances and the traditional *dop* system, in which farm laborers were paid, in part, with wine. Although the *dop* system has been outlawed since the 1920s, regular and heavy alcohol consumption persists in a high proportion (~30%) of women during pregnancy in this community (Jacobson et al. 2006a, 2008) despite numerous efforts to reduce pregnancy drinking. The Cape Coloured community, like those in Russia and the Ukraine with a very high prevalence of heavy drinking during pregnancy (e.g., Miller et al. 2006; Chambers et al. 2008) provides a unique opportunity to prospectively recruit large numbers of newborns with FAS, which would not be possible at a single U.S. site without screening tens of thousands of pregnant women.

In the Cape Town longitudinal study, 159 pregnant women were recruited at an antenatal clinic selected for its high prevalence of heavy maternal alcohol use (Jacobson et al. 2008). A timeline follow-back interview was administered to determine incidence and amount of drinking on a day-by-day basis during a typical 2-week period during early pregnancy (Sokol et al. 1985; Jacobson et al. 2002a). Volume was recorded for each type of beverage consumed each day and converted to absolute alcohol (AA). Any woman reporting a minimum of 14 drinks per week (1.0 oz AA/day) or at least two incidents of binge drinking (≥ 5 drinks) per month during the first trimester of pregnancy was invited to participate in the study. The next woman initiating antenatal care for whom gestation was within 2 weeks of the heavy drinking mother was also invited to participate, provided that she reported drinking < 7 drinks per week and did not binge drink. All the children were assessed for growth and FAS dysmorphology by expert dysmorphologists (HE Hoyme, LK Robinson, N Khaole) using a standard protocol (Hoyme et al. 2005). Based on clinical examination and prenatal alcohol interview, 18.2% of the children born to the heavy drinking mothers in this cohort met criteria for FAS, which is considerably higher than the expected four per 100 heavy drinkers in the U.S. (Abel 1995). An additional 23.2% met criteria for partial FAS (PFAS), while 58.6% of those born to heavy drinking mothers did not exhibit FAS facial anomalies. The high incidence of FAS likely reflects the exceedingly high concentrated pregnancy drinking in this community, which averaged 7.4 drinks/occasion on 3 days/week among the heavy drinking mothers.

Eyeblink Conditioning at 5 Years of Age

Two sessions of delay conditioning consisting of 50 trials each were administered to the children in the Cape Town longitudinal cohort on the same day about 2 h apart at age 5

years; a third session was administered the following day to those children who did not meet criterion for EBC in session 2 (Jacobson et al. 2008).

Children in all diagnostic groups exhibited a consistently high rate of unconditioned response to the air puff ($M=79.6\%$ of the trials) during all three sessions ($M\geq 74.7\%$ for each group, indicating that all groups could perceive the air puff and perform the eyeblink response to a similar extent. Table 1 presents the number of children who met criterion for conditioning in each of the three sessions. Prenatally exposed children were significantly less likely to meet the 40% CR criterion for conditioning than the controls. Not a single child of the 10 with FAS was conditioned as contrasted with 75.0% of the 20 controls. Performance in the other alcohol-exposed groups was also poor and was ordered by degree of alcohol exposure. By contrast, three of four non-exposed microcephalic children were conditioned by the second session, supporting the inference that the EBC deficit is specific to prenatal alcohol exposure and can provide a useful biobehavioral marker for the diagnosis of exposed children lacking the distinctive FAS dysmorphology. Although the IQ scores of the non-exposed microcephalic children ($M=80.7$) were similar to those of the children with FAS and PFAS ($M=79.7$), they met criterion for conditioning as readily as the controls, whose IQ scores ($M=89.4$) were significantly higher.

Figure 3a shows percent CRs for the first two sessions by diagnostic group. There was a significantly greater increase in CRs across sessions for the controls than for the exposed children, an effect that remained significant after IQ was included as a covariate. Estimated peak blood alcohol concentration (BAC, based on absolute alcohol per occasion, duration drinking per occasion (hours), and maternal weight; Markham et al. 1993), which is available for 30 mothers of the exposed children, averaged 242.2 mg/dL ($SD=150.1$). This BAC is considerably lower than the lower bound threshold of 300 mg/dL reported in heavily exposed rats who failed to condition (Stanton and Goodlett 1998), suggesting that either this endpoint is more sensitive for humans or the fetus is more sensitive when exposure occurs earlier in development than has been examined to date in the animal model.

Replication and Extension of the Eyeblink Conditioning Findings at School Age

Delay EBC was examined in a cross-sectional sample of 63 Cape Coloured children at mean age 11.3 years; trace conditioning, 1.5 years later in 32 of the same children at mean age 12.8 years (Jacobson et al. 2011b). As in our 5-year study, at each age, two sessions of 50 trials each were administered on the same day with two more sessions the next day, for children not meeting criterion for conditioning. The children in all of the diagnostic groups exhibited a consistently high rate of URs to the air puff in both the delay and trace conditioning sessions. The general absence of differences across groups in both the UR and startle responses to the tone suggests that prenatal alcohol exposure did not alter sensory processing of the tone or air puff or the ability to perform the eyeblink response. These findings are also consistent with the animal literature (e.g., Stanton and Goodlett 1998).

Acquisition of delay CRs was impaired in the alcohol-exposed children in this cross-sectional school-age sample, replicating the finding from the Cape Town longitudinal cohort at 5 years. Only 33.3% of the children with FAS and 42.9% of the heavily exposed children met criterion for delay conditioning, compared with 79.3% of the non- or light-exposed controls during the first two sessions (Table 2). Based on a logistic regression analysis, the children with FAS were 7.7 times more likely than controls to fail to meet criterion for conditioning on the delay task; the other heavy exposed group was 5.1 times more likely to fail.

Figures 3a and b show the acquisition curves for percentage CRs for each of the groups in 10-trial blocks for the first two 50-trial sessions of delay conditioning for the 5-year and

school-age samples. As seen in Fig. 3a, 5-year-old children in the control group showed a substantial increase in percent CRs at the beginning of Session 2, and their percent CRs remained consistently high throughout the second session. By contrast, there was no evidence of conditioning among the three alcohol-exposed groups across the ten blocks. A remarkably similar pattern is seen in the acquisition curves shown in Fig. 3b for the 11-year cohort. In both figures, there is virtually no overlap of the curves representing the performance of the nonexposed children with those representing the exposed children's performance. It should be noted that the effect of prenatal alcohol exposure on percent CRs persisted after control for IQ at both ages.

As expected, trace was more difficult than delay conditioning for all of the children but was particularly difficult for the alcohol-exposed groups (Table 2). Only 1 of 6 (16.7%) children with FAS and three of 14 (21.4%) of the heavily exposed children met criterion for trace conditioning, compared with two-thirds of the controls during the first two sessions. The six children with FAS were 10.0 times more likely to fail to meet criterion on the trace task compared with 12 controls, and the 14 heavily exposed children were 7.3 times more likely to fail to meet criterion.

Coffin et al. (2005) noted that one limitation of their study was the relative brevity of their conditioning protocol, which consisted of a single session, and raised the question of whether alcohol-exposed children would acquire the conditioned response if conditioning were given more extended training. This question was subsequently examined in the Cape Town cross-sectional study. Table 2 indicates the session during which each child first met the criterion of 40% CRs. Extended training was beneficial for some exposed children, who met criterion by the third or fourth session for delay conditioning, but both groups continued to perform markedly more poorly than the controls, 89.7% of whom were conditioned by the end of the third session. By contrast to the control children, two-thirds of whom met criterion for trace conditioning in two sessions, the majority of the exposed children who met criterion did not do so until Session 3 or 4. Even with prolonged training, only half as many of the FAS group met criterion for trace conditioning compared to controls, and percent CRs was much lower among the alcohol-exposed children than the controls.

Precision of timing of eyeblinks in delay conditioning was assessed by examining latency to CR onset during the CS tone-alone trials. As shown in Fig. 1, onset of the air puff in the delay task occurred 650 ms after the onset of the tone. The exposed children initiated their CRs significantly later than the controls (Jacobson et al. 2011b). Among the children with FAS, average CR onset during the tone alone trials occurred after the onset of when they had experienced the air puff during the paired trials. For the heavily exposed children, it occurred at the same time as the onset of the air puff, whereas among controls it occurred at or in anticipation of when the air puff would be expected. These findings are consistent with rodent studies, which have shown severe and permanent impairment of the production and timing of responses in eyeblink conditioning in alcohol exposed animals (Brown et al. 2008; Green et al. 2000; Stanton and Goodlett 1998). These deficits have been linked directly to cerebellar damage and reductions in estimated number of Purkinje cells in the developing rat brain (Goodlett et al. 1990; Thomas et al. 1998; Green et al. 2000; Dunty et al. 2001; Maier and West 2001). Thus, although some alcohol-exposed children can benefit from more extensive training, the data on precision of timing suggest that the quality of their CRs continues to be impaired.

These findings are consistent with the report by Coffin et al. (2005), who also found a reduced number and later onset of CRs in school-age, alcohol-exposed children with attention problems and dyslexia. In contrast to the alcohol-exposed children, Coffin et al.'s ADHD group produced CRs at a rate similar to normal controls, but these CRs were of

shorter latency, and the children with ADHD were less able to sustain the learned response. Frings et al. (2010) have reported similar patterns. Thus, despite the similarity in clinically assessed attentional problems, the alcohol exposed and ADHD children exhibited distinctive EBC patterns, which Coffin et al. suggest are likely to reflect impairment of distinct cerebellar regions. The most consistent cerebellar difference in children with ADHD is smaller cerebellar vermis (Berquin et al. 1998; Castellanos et al. 2002) but no difference in cerebellar hemispheres, a region in which rats developmentally exposed to alcohol show severe reductions in estimated numbers of Purkinje cells (Goodlett et al. 1990; Thomas et al. 1998). Coffin et al. also noted that these EBC differences may account for the poorer response by alcohol-exposed children to the standard pharmacological interventions for reducing attentional disturbances that have been found to be effective with children with ADHD. They cite Anderson et al. (2002)'s finding that methylphenidate has a beneficial effect on cerebellar vermis but not adjacent cerebellar hemispheres as further support for this interpretation. Thus, delay EBC may discriminate between individuals with different disorders involving cerebellar anomalies who exhibit similar behavioral problems.

Coffin et al. (2005) also examined EBC in a group of children with dyslexia. Like the alcohol-exposed children, the dyslexia group produced few CRs, suggesting a possible similarity in the cerebellar impairment in the two groups. The children in the alcohol-exposed group also had notable problems in reading. However, in a pilot study comparing college-aged dyslexics and normal controls, there was no evidence of conditioning in dyslexics even after five consecutive days of training (Coffin and Boegle 2000), whereas we found that many of the alcohol-exposed children benefited from extended training (Jacobson et al. 2011b). It should be noted, nonetheless, that although more alcohol-exposed children met criterion for conditioning (40% CRs) after four sessions, the impairment persisted in the children with FASD since CRs had increased to even higher levels in the control children.

The Coffin et al. (2005) and our Cape Town studies used only one short delay interstimulus interval. It has been suggested that EBC paradigms with different interstimulus intervals can provide additional information regarding timing deficits (e.g., Koekkoek et al. 2003; McGlinchey-Berroth et al. 1999). If there is a cerebellar-dependent timing deficit, it should appear at various time intervals. Frings et al. (2010) used a delay EBC paradigm with two different interstimulus intervals to detect possible timing deficits in patients with ADHD. Onset and peak of the CR in the long delay condition occurred earlier in the ADHD group than in controls, but there was no difference in their performance in the short delay condition. This discrepancy suggests that the poorer long delay EBC performance in the ADHD group is likely due to a noncerebellar problem. By contrast, the alcohol-exposed children in our study exhibited timing problems in both the short delay condition and the trace condition, which used a long CS-US interval (see Fig. 1), suggesting that alcohol-related cerebellar damage is implicated in their EBC impairment (Jacobson et al. 2011b). Moreover, these children's late responses also distinguish them from patients with cerebellar lesions, who tend to respond too early (Frings et al. 2010), which may explain the capacity of some of the alcohol-exposed children to benefit from extended training.

Neuroimaging Studies of FASD Relating to Eyeblink Conditioning

The earliest autopsy studies reporting damaging effects of heavy prenatal alcohol exposure identified errors in cell migration, as well as agenesis or thinning of the corpus callosum, and anomalies in the cerebellum and brain stem (Jones and Smith 1973; Clarren 1977; Clarren and Smith 1978). In a review of four studies, cerebellar dysgenesis was found in 10 of 16 FAS autopsies (Clarren 1986). Cerebellar abnormalities are also found in rats and mice exposed to alcohol prenatally (Miller and Robertson 1993; Maier et al. 1999) with significantly fewer Purkinje cells and lower cerebellar weight to body weight ratios (Bauer-Moffett and Altman 1977; Bonthius and West 1991; Marcussen et al. 1994; Goodlett et al.

1990). In the only study to perform a comprehensive morphometric analysis of the four major cortical lobes, cerebellum, and principal subcortical regions, Archibald et al. (2001) found a significant deficit in total brain volume, with disproportionately smaller volumes in cerebellum, parietal lobe, and caudate nucleus, including a 15% smaller cerebellar volume in individuals with FAS. Analyses comparing cerebrum and cerebellum suggested that cerebellar hypoplasia may exceed cerebral hypoplasia.

A growing body of evidence suggests that white matter is a specific target of alcohol teratogenesis. Archibald et al. found proportionately less cerebral white matter in their FAS group, suggesting an effect on myelination that has also been observed in ethanol-exposed animals (Bichenkov and Ellingson 2001; Zoeller et al. 1994). White matter lesions have also been observed in preterm infants with heavy prenatal alcohol exposure (Holzman et al. 1995). These lesions were also seen in fetal alcohol exposed sheep (Watari et al. 2006). Studies with fetal alcohol exposed rodents have reported decreases in axon size, increased packing density, and thinner myelin sheaths (Miller and Al-Rabiaai 1994), as well as abnormalities in the oligodendrocytes that produce the myelin sheath (Chiappelli et al. 1991; Guerri et al. 2001). As detailed below, we have recently identified fetal alcohol-related microstructural deficits in the cerebellar peduncles, large bundles of myelinated nerve fibers that connect the cerebellum to the brainstem and play a major role in the EBC circuit (Spottiswoode et al. in press).

Several considerations make eyeblink conditioning an excellent candidate to serve as a biobehavioral marker of fetal alcohol effect, including the high sensitivity of both trace and delay conditioning to prenatal alcohol exposure, the detailed information available regarding the neural structures and pathways that mediate this elemental form of learning, and the evidence of the sensitivity of cerebellum and white matter to alcohol exposure in utero. As a biobehavioral marker, this paradigm has considerable potential to advance understanding of the neural mechanisms that mediate fetal alcohol effects. In a recent DTI study focusing on the cerebellar peduncles, we found a dose-dependent inverse relation between prenatal alcohol exposure and fractional anisotropy (FA) in a region of the left middle peduncle (Spottiswoode et al. in press). Higher FA in that region was also related to better performance on trace EBC. In a multiple regression analysis, the effect of prenatal alcohol exposure on trace EBC was reduced from $r = -.61$ to $\beta = -.28$ when FA in that region was entered in the analysis—a decrease that was statistically significant. That substantial decrease suggests that the alcohol-related trace EBC deficit is mediated, in part, by microstructural impairment in the left middle peduncle. By contrast, the regression coefficient relating prenatal alcohol to trace EBC was virtually unchanged when total brain volume and cerebellar volume were each added to the analysis, indicating that brain volume per se does not mediate this deficit. The effect of alcohol exposure on trace conditioning was, however, significantly mediated by cerebellar white matter volume.

Number Processing

Among the broad range of cognitive deficits, arithmetic is a particularly sensitive endpoint of fetal alcohol exposure (Rasmussen and Bisanz 2009). In assessments of academic achievement conducted in the moderately exposed Seattle 500 cohort, arithmetic was the domain most strongly related to maternal drinking in pregnancy at both 7.5 (Streissguth et al. 1990) and 14 years (Streissguth et al. 1994). Similarly, in the Atlanta cohort, arithmetic was the academic subtest of the Kaufman Assessment Battery for Children (K-ABC) most strongly related to prenatal alcohol exposure at 6 years (Coles et al. 1991), and arithmetic and digit span were the Wechsler IQ subtests most strongly related to pregnancy drinking in the Detroit longitudinal cohort at 7.5 years (Jacobson et al. 2004). In a study comparing adolescents with alcohol-related dysmorphic features with a group of children in special

education classes, the special education students were more impaired in basic reading and spelling, whereas the alcohol-exposed adolescents were more impaired in mathematics and mathematical reasoning (Howell et al. 2006). In the Pittsburgh cohort, prenatal alcohol exposure was related to poorer performance on standardized tests of reading, spelling, and arithmetic, but only the effect on arithmetic was dose-dependent and remained significant after statistical adjustment for IQ (Goldschmidt et al. 1996).

The children in the Detroit cohort were administered reaction time tasks at 7.5 years using a Sternberg (1966) paradigm to assess four domains of cognitive processing—short-term memory scanning, mental rotation, directional discrimination, and number comparison (Burden et al. 2005b). In the Sternberg paradigm, the slope of the reaction times across task items of increasing difficulty is used to assess cognitive processing speed and efficiency in a given domain, controlling for influences from other, unrelated domains involved in performance of the task, including response organization and execution and motor speed. After control for potential confounders, prenatal alcohol exposure was related to poorer cognitive processing efficiency only on the number comparison task. Kopera-Frye et al. (1996) compared adults with FAS or fetal alcohol effects with healthy controls matched for age, gender, and education level on a series of number processing tasks. Although the performance of the alcohol-exposed adults on number reading and dictation was not affected, they performed significantly more poorly on tasks involving arithmetic calculation and proximity judgment. In a sample of adults diagnosed with FAS who performed poorly on standardized tests of reading, spelling, and arithmetic, arithmetic scores were significantly lower than scores on reading or spelling (Kerns et al. 1997).

Brain Mechanisms Mediating Number Processing

Brain lesion and neuroimaging studies have identified two distinct functional neural networks relating to number processing—(a) a core quantity system, in which numerical quantity (magnitude and distance) is represented in a language-independent format, possibly resembling a number line (Dehaene et al. 2004); and (b) mental calculation, involving manipulation of verbally-encoded numbers and verbally-stored knowledge (e.g., arithmetic facts) (Menon et al. 2000; Zago et al. 2001). Magnitude comparison, the ability to evaluate relative quantities, has been shown to be mediated primarily by activity in the anterior portion of the horizontal section of the intraparietal sulcus (HIPS) (Dehaene et al. 2003; Eger et al. 2003; Pinel et al. 2004). It emerges early in development (Meintjes et al. 2010a; Wynn et al. 2002;) and is clearly evident at 4–5 years of age (Temple and Posner 1998; Cantlon et al. 2006). The anterior HIPS is activated by the representation of semantic information about magnitude, whether presented as Arabic numbers, sequences of words, or analogically (e.g., by numbers of dots) (Dehaene and Cohen 1995; Naccache and Dehaene 2001). Activations in the anterior HIPS increase as the complexity of the calculation increases; for example, in calculations entailing a greater number of operands (Menon et al. 2000).

Calculation involves recruitment of a fronto-parietal number processing network that includes the anterior HIPS (Chochon et al. 1999; Simon et al. 2002) and the angular gyrus in the parietal lobe in interaction with a frontal executive brain system (Zago et al. 2008) not specific to number processing. The frontal executive system mediates the integration and management of numerical operations in working memory, response decision and execution, and error monitoring (Gruber et al. 2001; Menon et al. 2000). Thus, intensity of inferior frontal activation is directly proportional to the time pressure imposed during a number processing task rather than the complexity of the numerical calculation per se (Menon et al. 2000).

In school-age children, number processing is associated with activation of a similar parietofrontal network to that seen in adults, including the anterior HIPS (Cantlon et al. 2006; Kaufman et al. 2006; Meintjes et al. 2010a), although activations are also seen in the posterior medial frontal cortex (pmFC; Kucian et al. 2006; Kaufman et al. 2006) and the left precentral sulcus (Kawashima et al. 2004). In their meta-analysis, Ridderinkhof et al. (2004) showed that activations of the pmFC, which includes the anterior cingulate cortex, are associated with pre-response conflict, decision uncertainty, and error detection. The activation in the precentral sulcus commonly seen in number processing in children overlaps with the premotor strip at the coordinates of finger representation. Given that finger counting is a spontaneous numerical learning strategy that has been observed cross-culturally (Butterworth 1999), it has been suggested that a fronto-parietal finger movement network may develop in the skilled user to become a substrate that mediates numerical knowledge (Pesenti et al. 2000; Zago et al. 2001).

Magnitude Comparison as a Mediator of Fetal Alcohol-Related Effects on Number Processing

In a recent study, Jacobson et al. (2011a) have shown that a specific deficit in the ability to represent and manipulate quantity appears to play a critical role in the poor arithmetic performance seen in FASD. A 224-item, 7-subtest, computer-based Number Processing test, developed in collaboration with S. Dehaene, was administered to 262 adolescents from the Detroit Longitudinal Cohort at 14 years. Prenatal alcohol exposure was significantly related to poorer performance on one subtest after control for confounding, Approximate Subtraction, and was most strongly related to Number Comparison (“Which number is larger?”) and Proximity Judgment (“Which of two numbers is closer to a third?”). A factor analysis of the seven subtests yielded two factors, one for “Calculation,” which included Exact Addition, Subtraction, and Multiplication and Approximate Addition and Subtraction; the other “Magnitude Comparison,” which comprised Number Comparison and Proximity Judgment. Prenatal alcohol exposure was significantly related to both the Calculation and Magnitude Comparison composite measures.

Multiple regression analysis showed that the relation of prenatal alcohol exposure to Calculation was fully mediated by its relation to Magnitude Comparison since the entry of Magnitude Comparison into the regression reduced the effect of alcohol on Calculation from $r = -.14$ to $\beta = .03$ (Fig. 4). The Sobel test showed that the coefficients for the path indicating that the effect of prenatal alcohol exposure on Calculation is mediated via Magnitude Comparison was highly significant, $z = -3.54$, $p < .001$. This finding suggests that a specific deficit in the ability to represent and manipulate quantity plays a critical role in the poorer arithmetic achievement frequently seen in FASD and may be a core deficit of the disorder.

Attention deficit hyperactivity disorder (ADHD) was evaluated in the children in the Detroit cohort at 7.5 and 14 years of age based on parent, teacher, and examiner ratings on check lists of behavioral symptoms that constitute the criteria for a DSM diagnosis (Burden et al. 2010; Jacobson et al. 2011a). Using this approach, 32.8% of the children met criteria for a diagnosis of ADHD, confirming the co-morbidity of this disorder with FASD (Mick et al. 2002; Fryer et al. 2007). Effects of ADHD on number processing were compared with effects of prenatal alcohol exposure for the sample as a whole, including children who were co-morbid for prenatal alcohol exposure and ADHD in both sets of analyses. ADHD was related to poorer performance on all seven number processing subtests, after control for confounders but, by contrast to the alcohol-exposed children, the associations were markedly stronger with the exact and approximate calculation subtests and the Calculation composite than with Number Comparison, Proximity Judgment, or the Magnitude Comparison composite. Moreover, the relation of ADHD to the exact and approximate calculation subtests was markedly reduced when IQ was added at the last step of the

multiple regression analysis, suggesting that effects of ADHD on aspects of calculation not specific to the representation of number, such as, attention and executive function, mediate the poorer number processing seen in that disorder. The hypothesis that IQ mediates the effect of ADHD on the two composite measures was evaluated statistically using the Sobel test. The relation of ADHD to both composites was significantly mediated by IQ; for Calculation, $z=-2.75$, $p<.01$, for Magnitude Comparison, $z=-1.96$, $p<.05$. By contrast, IQ did not mediate the effect of prenatal alcohol exposure on either Calculation, $z=-1.37$, or Magnitude Comparison, $z=-1.28$, both p 's $>.15$, presumably because the alcohol-related arithmetic deficits are not mediated by deficits in higher order cognitive processing.

The finding that Magnitude Comparison is the principal aspect of number processing affected by fetal alcohol exposure has been replicated in a study of children from the Cape Town cross-sectional cohort, who were evaluated at a mean age of 10.4 years ($SD=1.2$). A confirmatory factor analysis specifying two factors yielded the same pattern observed in Detroit—exact and approximate addition, subtraction, and multiplication loaded on a Calculation factor; Number Comparison and Proximity Judgment, on a Magnitude Comparison factor. Whereas prenatal alcohol exposure was unrelated to the Calculation composite score after control for confounders ($\beta=-.06$, $p>.20$), it was significantly related to the Magnitude Comparison Composite ($\beta=-.25$, $p<.05$) after control for socioeconomic status, postnatal lead exposure, and child age at testing. Thus, these data provide additional evidence identifying mental representation of relative quantity as the principal aspect of number processing adversely affected by fetal alcohol exposure.

Neuroimaging Studies Relating to Number Processing in FASD

A functional MRI (fMRI) study of 18 healthy control children from the Cape Town cross-sectional cohort (Meintjes et al. 2010a) confirmed that mental representation of quantity and distance is mediated by activation of the intraparietal sulcus, as has been extensively documented in adults (Dehaene et al. 2003; Eger et al. 2003). In an fMRI study of the Atlanta cohort using a subtraction task, young adults with fetal alcohol-related dysmorphology showed less activation in the inferior parietal and other math-related regions compared with normal controls (Santhanam et al. 2009). In a comparison of children diagnosed with FAS or PFAS with healthy controls in the Cape Town cross-sectional cohort, the anterior HIPS activation during Proximity Judgment was right lateralized in the control children as it is in adults (Chochon et al. 1999) but left lateralized in the FAS/PFAS group (Meintjes et al. 2010b). By contrast, the anterior HIPS activation was left lateralized during Exact Addition in the control group as would be expected for verbally-mediated addition problems but bilateral in the children with FAS and PFAS. In addition, by contrast to the control group, the children with FAS and PFAS failed to exhibit an activation in the left precentral sulcus, which is involved in finger counting (Butterworth 1999) and believed to function as a substrate that mediates numerical knowledge in normal children and adults (Pesenti et al. 2000; Zago et al. 2001).

Whereas control children exhibited highly focused activations of the intraparietal sulcus during both tasks, the alcohol-exposed children exhibited a highly diffuse parietal activation during Proximity Judgment, which included the left and right angular gyrus and posterior cingulate/precuneus (Meintjes et al. 2010b). Data from several studies suggest that the left angular gyrus and adjacent frontal perisylvian areas are more likely to be activated for number processing problems requiring more extensive verbal mediation (Dehaene et al. 2003; Zago et al. 2001; Venkatraman et al. 2005). Gruber et al. (2001) found increased activations in the precuneus/posterior cingulate region for simple tasks requiring arithmetic fact retrieval and note that this region is activated in semantically cued word retrieval facts (Fletcher et al. 1996). The activations of the left angular gyrus, precuneus/posterior cingulate, and left anterior HIPS suggest that the exposed children may rely more on verbal

recitation of the numbers and/or verbally-mediated subtraction operations to solve the PJ problems, instead of the nonverbal quantity comparison processing that has been shown to be mediated by the right anterior HIPS (Chochon et al. 1999).

During Exact Addition, the control children in the Cape Town cohort (Meintjes et al. 2010b) exhibited activations of a fronto-parietal network similar to that linked to number processing in adults (Chochon et al. 1999). The exposed children, by contrast, exhibited more diffuse and wide-spread activations, including the cerebellar vermis and cortex, which have been found to be activated in adults engaged in particularly challenging number processing problems. This recruitment of a broader range of regions to perform these relatively simple addition problems suggests a deficit in functional integration of the neural circuitry that is most efficient for the manipulation and processing of numerical quantity. The functional deficits in the parietal region seen in the exposed children in these neuroimaging studies are consistent with evidence from structural MRI studies reporting alcohol-related impairment in the parietal region, including disproportionately smaller lobular volume (Archibald et al. 2001) and less gray matter and cortical thickness, and shape anomalies in the parietal and temporal regions (Sowell et al. 2001b, 2002, 2008a).

Infant Numerosity

Characterization of FASD during development and the design of effective early interventions require identification of the fundamental and earliest emerging cognitive processes that are affected by prenatal alcohol exposure—processes that provide the basis for the higher-order deficits in cognitive processing seen in affected children and adults. Recent evidence suggests that early precursors of alcohol-related impairment in magnitude comparison can be detected already in infancy. Infants can discriminate small precise numerosities (see reviews in Mix et al. 2002; Butterworth 2005). Wynn (1992) has shown that infants as young as 5 months of age can discriminate between correct displays (e.g., $1+1=2$) and errors (e.g., $1+1=1$) in simple arithmetic problems involving small numbers of items. When the number of puppets displayed does not agree with the number previously seen being placed on the stage, infants look longer at the display than when the number agrees with their expectations (Wynn 1995, 1996; Wynn and Chiang 1998). When the Wynn paradigm was administered to the Cape Town Longitudinal Cohort in infancy, nonexposed infants (as predicted) looked longer at puppet displays that were incongruent with their expectations (Jacobson et al. 2002b). By contrast, alcohol-exposed infants' looking time was the same to both the expected and unexpected displays, indicating a failure to discriminate among these small numerosities. Looking time differences (incongruent minus congruent) on the Wynn paradigm at 6 and 13 months were subsequently converted to z-scores and averaged to provide a composite measure of numerosity performance. Numerosity on the Wynn paradigm predicted performance on the Number/Quantity and Digit Span subtests of the Junior South African Intelligence Scale (J-SAIS), $r=.34$, $p<.01$, and $r=.25$, $p<.05$, respectively, at 5 years but was unrelated to the J-SAIS subtests assessing vocabulary and fine motor function, $r's=.10$ and $-.01$, both $p's>.20$, indicating discriminant predictive validity for this infant numerosity paradigm.

Conclusions

Whereas biomarkers of exposure, such as FAEE metabolites of alcohol in meconium, can provide verification of maternal reported alcohol consumption during pregnancy, biobehavioral markers of effect are needed to help identify which exposed children are adversely affected. Classical EBC and magnitude comparison provide good examples of biobehavioral markers in that the pattern of effects seen on these endpoints appears to be relatively specific to FASD and is mediated by neural processes and pathways that have been well characterized in the animal model and/or neuro-imaging literatures. Working

memory is a third endpoint that could be examined as a potential biobehavioral marker since it is seen in FASD after statistical adjustment for IQ (Burden et al. 2005a, b, O'Hare et al. 2009; Diwadkar et al. in press) and its neural substrates have been extensively documented (Wager and Smith 2003; Kirschen et al. 2005). Verbal encoding, recall, and recognition discrimination, as assessed on the California Verbal Learning Test, may also warrant consideration as biobehavioral markers since they have been linked to FASD (e.g., Mattson et al. 1996; Mattson and Roebuck 2002; Willford et al. 2004; O'Leary et al. in press) and to deficits in cerebellar vermis and corpus callosum (Sowell et al. 2001a, b, 2008a, b).

Although the comparison with ADHD demonstrates that magnitude comparison is a relatively specific endpoint for prenatal alcohol exposure, this outcome is less sensitive than eyeblink conditioning. To examine sensitivity, we used a cut-off of 1 standard deviation (SD) below the mean in the control group to indicate poor performance. Whereas 16.4% of the controls in the Detroit study performed at more than 1 SD below the mean, almost twice as many of the exposed adolescents (27.8%) performed below this cut-off. In Cape Town, 12.9% of the controls performed at more than 1 SD below the mean, and almost three times as many exposed children (35.3%) performed poorly. By contrast, as reported above, all of the children with FAS and 63.8% of the other heavily exposed children failed to meet criterion for eyeblink conditioning at 5 years and at school age, 66.7% of the children with FAS and 57.2% of the other heavily exposed children failed to reach criterion within two sessions. Given that some children with prenatal alcohol exposure may be protected from the effects of alcohol (e.g., Jacobson et al. 2006a, b) and not exhibit adverse effects, these biobehavioral markers can be useful in identifying which exposed children are affected and which appear to be spared. Taking performance on both these biobehavioral markers into account can further improve identification of alcohol-exposed children. In the school age Cape Town cohort, 58.8% of the alcohol-exposed children failed to meet criterion for delay EBC and 35.3% for magnitude comparison, but 71.1% of the exposed children were identified on at least one of these biobehavioral markers.

It is not clear that either of these biobehavioral markers should be used routinely in the screening of alcohol-related disorders; the sensitivity of magnitude comparison is limited, and the EBC assessment is difficult to administer and requires two 15-minute sessions given at least 2–3h apart. Nevertheless, where FAS, PFAS, or ARND is suspected, EBC and magnitude comparison could generate valuable information as part of an in-depth clinical examination of the child by an interdisciplinary team, including a geneticist to rule out other disorders and examiners with expertise to conduct conditioning trials and psychometric testing. If the child is known to be alcohol exposed or even if no history of maternal alcohol use available but the child has some features or behaviors suggestive of fetal alcohol exposure, failure to meet criterion on the EBC assessment would provide an additional strong indicator of likely damage to the cerebellar-brain stem circuitry frequently affected in FASD; poor performance on magnitude comparison, of likely damage to the relevant fronto-parietal circuitry.

This in-depth clinical assessment, combined with other biobehavioral markers of FASD, would provide a more definitive diagnosis than a low IQ score, which is neither specific to this disorder nor sufficiently sensitive, particularly when moderate rather than heavy alcohol exposure is involved (Jacobson et al. 2004). As indicated earlier, EBC and magnitude comparison are both unrelated to IQ performance and can, therefore, be used to detect alcohol-related impairment in children performing in the normal IQ range. EBC performance is also unrelated to socioenvironmental background or verbal competence, which are often confounded with IQ deficits. Similarly, as noted above, ADHD and arithmetic problems are often co-morbid in alcohol-exposed children, but evidence of specific problems in magnitude representation increases the likelihood that fetal alcohol

exposure is involved. Assessments of performance on biobehavioral markers can also provide valuable information regarding specific domains of function in need of remediation. Thus, when a child whose mother is suspected of heavy alcohol use during pregnancy presents with ADHD-like symptoms, assessment of magnitude comparison can aid in a differential diagnosis that may lead to a different pharmacological treatment, since methylphenidate and other psychostimulants often prescribed for ADHD have not proven to be as effective with children with FASD (see Kodituwakku and Kodituwakku 2011). The Math Learning Experience (MILE) intervention developed by Kable and Coles (Kable et al. 2007) is particularly well suited for remediating the specific fetal alcohol-related deficits in magnitude comparison.

An important strength of these biobehavioral markers is that they have been linked in cognitive neuroscience studies to specific neural processes that can be assessed in infancy. They can, therefore, be used to evaluate the efficacy of novel prenatal interventions (e.g., supplementation with micronutrients) several months or years before appropriate neurobehavioral assessments can be administered and to validate new diagnostic procedures (e.g., facial measurements from 3-D images; Moore et al. 2007; Klingenberg et al. 2010). They can also provide baseline data for evaluating novel postpartum interventions (e.g., therapeutic motor training developed by Klintsova et al. 1998; Jirikowic et al. 2010), whose efficacy for reversing or mitigating alcohol effects can be assessed by measurement again at the conclusion of the intervention. As we have emphasized, the neural circuitry mediating both these biobehavioral markers is well understood. Future studies of fetal alcohol effects on specific components of this neural circuitry have considerable potential for providing important information regarding the pathophysiology of FASD, which can, in turn, contribute to the development of treatments that are better targeted to the specific deficits that characterize this disorder.

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Fig. 1.
Schematic diagram of trial epochs used in delay and trace conditioning

Fig. 2.
Simplified diagram of the cerebellar memory circuit of the essential circuitry for classical eyeblink conditioning (adapted from Christian and Thompson 2003)

Fig. 3.

Percent conditioned responses in each block by fetal alcohol spectrum disorders diagnostic group (**a**) at 5 years and (**b**) at 11 years. Error bars are standard error of the mean. Blocks 1 to 5 = Session 1; Blocks 6 to 10 = Session 2 ($N=81$ (40 males; 37 females) at 5 years; $N=63$ (23 males; 40 females) at 11 years)

Fig. 4. Path model examining the degree to which the relation of prenatal alcohol exposure to the Calculation composite score is mediated by Magnitude Comparison

Eyeblink conditioning by session during which child first met criterion at 5 years. Values are number of children (%) who met the criterion of at least 40% conditioned responses (CR) in a session. Each child is shown in the session in which s/he first met the CR criterion

Table 1

	Alcohol-exposed			Nonexposed		Total
	FAS	Partial FAS	Heavy exposed	Control		
Session 1	0 (0.0)	2 (11.1)	3 (10.3)	4 (20.0)	9 (11.7)	
Session 2	0 (0.0)	3 (16.7)	2 (6.9)	9 (45.0)	14 (18.2)	
Session 3	0 (0.0)	1 (5.6)	6 (20.7)	2 (10.0)	9 (11.7)	
Total conditioned	0 (0.0)	6 (33.3)	11 (37.9)	15 (75.0)	32 (41.6)	
Total <i>N</i>	10	18	29	20	77	

Table 2

Number (%) of children meeting criterion for delay and trace conditioning at school age by diagnostic group and session during which child first met criterion. Values are number of children (%) who met the criterion of at least 40% conditioned responses (CR) in a session

	Alcohol-exposed		Nonexposed Control	Total
	FAS	Heavy exposed		
Delay conditioning				
Session 1	0 (0.0)	6 (21.4)	12 (41.4)	18 (28.6)
Session 2	2 (33.3)	6 (21.4)	11 (37.9)	19 (30.2)
Session 3	1 (16.7)	4 (14.3)	3 (10.3)	8 (12.7)
Session 4	1 (16.7)	1 (3.6)	0 (0.0)	2 (3.2)
Total conditioned	4 (66.7)	17 (60.7)	26 (89.7)	47 (74.6)
Total N	6	28	29	63
Trace conditioning				
Session 1	1 (16.7)	2 (14.3)	3 (25.0)	6 (18.8)
Session 2	0 (0.0)	1 (7.1)	5 (41.7)	6 (18.8)
Session 3	1 (16.7)	4 (28.6)	0 (0.0)	5 (15.6)
Session 4	0 (0.0)	1 (7.1)	0 (0.0)	1 (3.1)
Total conditioned	2 (33.3)	8 (57.1)	8 (66.7)	18 (56.3)
Total N	6	14	12	32