Research

Ontogeny of contextual fear memory formation, specificity, and persistence in mice

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Pinpointing the precise age when young animals begin to form memories of aversive events is valuable for understanding the onset of anxiety and mood disorders and for detecting early cognitive impairment in models of childhood-onset disorders. Although these disorders are most commonly modeled in mice, we know little regarding the development of learning and memory in this species because most previous studies have been restricted to rats. Therefore, in the present study, we constructed an ontogenetic timeline of contextual fear memory ranging from infancy to adulthood in mice. We found that the ability of mice to form long-term context-shock associations emerged ~13–14 d of age, which is several days earlier than previously reported for rats. Although the ability to form contextual fear memories remained stable from infancy into adulthood, infant mice had shorter-lasting memories than adolescent and adult mice. Furthermore, we found that mice subjected to fetal alcohol exposure showed a delay in the developmental emergence of contextual fear memory, illustrating the utility of this ontogenetic approach in detecting developmental delays in cognitive function stemming from maladaptive early life experience.

[Supplemental material is available for this article.]

Mental illnesses are increasingly recognized as chronic disorders of the young (Insel and Fenton 2005). Many mental disorders—particularly those involving anxiety and lack of impulse control—emerge during childhood or adolescence and persist throughout the lifespan, with earlier onset associated with longer delays to initial treatment, increased comorbidity, and poorer functional outcomes (Kessler et al. 2007; McGorry et al. 2011). Timely diagnosis and treatment of these disorders, therefore, can reduce their severity and prevalence, thereby lessening the burden of mental illness on the individual, family, and society (de Girolamo et al. 2012).

In the study of the etiology of mental disorders, animal models are vital tools. Pinpointing the precise age when animals begin to form memories of aversive events can be valuable for understanding the onset of anxiety and mood disorders stemming from maladaptive early life experience (Pine 2009; Bale et al. 2010; Marco et al. 2011) and for detecting early cognitive impairment in models of autism, attention deficit/hyperactivity disorder, and fetal alcohol syndrome (Schneider et al. 2011; Kaffman and Krystal 2012). During infancy and adolescence, the progressive growth and refinement of neural circuitry supporting sensation and perception (Bourne 2010; Froemke and Jones 2011), cognition (Benes et al. 2000; Dumas 2005), and emotion (Braun 2011) gradually enables young rodents to begin learning about their surroundings. For example, around 17 d of age, rats start to form memories of contexts they encounter (Brasser and Spear 2004; Yap and Richardson 2005; Foster and Burman 2010), and by 23 d of age, they can associate those contexts with the occurrence of aversive events (Rudy 1993; Rudy and Morledge 1994; Raineki et al. 2010; Schiffino et al. 2011).

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Because of the ease of targeted genetic manipulation, mice have become the most prevalent animal models of mental disorders. However, apart from a few mouse studies (e.g., Paylor et al. 1996; Hefner and Holmes 2007; Ito et al. 2009), nearly all studies on the development of learning and memory have been restricted to rats. Because the two species differ in terms of synaptic architecture, sensory and motor function, and performance in behavioral tasks (Whishaw et al. 2001), knowledge pertaining to the ontogeny of learning and memory in rats may not directly transfer to studies employing mice. The primary aim of the present study, therefore, was to construct an ontogenetic timeline of memory in mice using contextual fear conditioning, a well-characterized aversive learning paradigm (Anagnostaras et al. 2001; Rudy et al. 2004). Furthermore, although maladaptive early life experience is often shown to produce cognitive impairments that are evident long after the initial trauma (Sullivan et al. 2006; McClelland et al. 2011), less research has focused on whether early adversity delays the initial onset of learning and memory in young animals. Thus, our second aim was to determine whether an ontogenetic timeline of contextual fear memory could be used to detect developmental delays resulting from one type of adverse early life experience—fetal exposure to alcohol.

Results

Memory formation

We investigated the ontogeny of contextual fear memory formation by placing infant (P13, P14, P15, P16, or P17), adolescent (P30), or adult (P60) mice in a context and delivering three foot shocks (shock group). Mice were returned to the context 24 h after training to test for long-term memory of the context-shock association. To control for differences among ages in spontaneous freezing, age-matched groups of mice were exposed to the context, but no foot shocks were delivered (no shock group).

We found that, across all ages tested, mice that were shocked in the context showed high levels of freezing upon return to the context 24 h later. High levels of freezing were also observed among infant mice that were not shocked, although this spontaneous freezing steadily diminished with age (age × shock interaction $P < 0.001$) (Fig. 1A; Supplemental Table S1). At P13, shocked and nonshocked mice showed similar levels of freezing. From P14 onward, however, shocked mice consistently froze more than nonshocked mice (P14 $P < 0.001$, P15 $P = 0.005$, P16 $P = 0.001$, P17 $P < 0.001$, P30 $P < 0.001$, P60 $P < 0.001$).

To further examine the developmental timeline of contextual fear memory formation, we analyzed minute-by-minute changes in freezing during training at each age. We found that at P13, the delivery of foot shocks increased locomotor activity, resulting in less post-shock freezing compared to mice that were not shocked (shock × minute interaction $P = 0.004$, minute 3 $P = 0.004$) (Fig. 1B). At P14, foot shocks did not affect freezing (Fig. 1C). However, starting at P15 and continuing through adolescence and into adulthood, the delivery of foot shocks consistently resulted in more post-shock freezing compared to nonshocked mice (P15 shock × minute interaction $P < 0.001$, minute 4 $P = 0.001$, minute 5 $P = 0.005$ [Fig. 1D]; P16 shock × minute interaction $P < 0.001$, minute 3 $P = 0.031$, minute 4 $P = 0.001$, minute 5 $P < 0.001$ [Fig. 1E]; P17 shock × minute interaction $P < 0.001$, minute 3 $P = 0.008$, minute 4 $P = 0.002$, minute 5 $P < 0.001$ [Fig. 1F]; P30 shock × minute interaction $P < 0.001$, minute 5 $P < 0.001$ [Fig. 1G]; P60 shock × minute interaction $P < 0.001$, minute 4 $P = 0.012$, minute 5 $P < 0.001$ [Fig. 1H].)

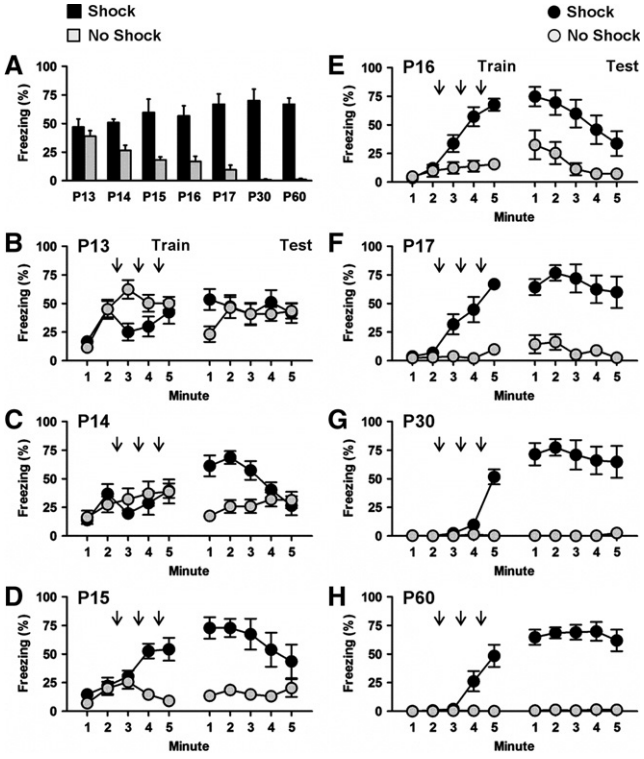

Figure 1. Contextual fear memory formation. Infant, adolescent, or adult mice were placed in a context, where they either received three unsignaled foot shocks (denoted by a downward arrow [↓]; shock group; P13 $n = 8$, P14 $n = 8$, P15 $n = 7$, P16 $n = 8$, P17 $n = 8$, P30 $n = 8$, P60 $n = 8$) or did not receive foot shocks (no shock group; P13 $n = 8$, P14 $n = 8$, P15 $n = 7$, P16 $n = 8$, P17 $n = 8$, P30 $n = 8$, P60 $n = 8$). Memory for the context-shock association was tested 24 h after training. (*A*) During the test, shocked mice showed high levels of freezing regardless of age, whereas nonshocked mice showed a gradual decline in spontaneous freezing with age. Minute-by-minute examination of freezing during training (*left* sides of graphs) and testing (*right* sides of graphs) revealed that P13 mice (*B*) exhibited negligible evidence of contextual fear memory. At P14 (*C*) and all ages thereafter—P15 (*D*), P16 (*E*), P17 (*F*), P30 (*G*), and P60 (*H*)—mice exhibited clear evidence of contextual fear memory, with shocked mice freezing more than nonshocked mice during the test.

We also analyzed minute-by-minute changes in freezing during testing at each age. At P13, both shocked and nonshocked mice exhibited high levels of freezing, with no significant differences between groups (Fig. 1B). At P14, shocked mice froze more than nonshocked mice during the first few minutes of the test (shock × minute interaction $P < 0.001$, minute 1 $P = 0.001$, minute 2 $P < 0.001$, minute 3 $P = 0.008$ [Fig. 1C]), showing a decline in freezing across minutes (minute main effect within shocked group, $F_{(4,28)} = 4.64$, $P = 0.005$). At P15 and P16, shocked mice also showed a decline in freezing across minutes (P15 minute main effect within shocked group, $F_{(4,24)} = 3.64$, $P = 0.019$ [Fig. 1D]; P16 minute main effect within shocked group, $F_{(4,28)} = 5.43$, $P = 0.002$ [Fig. 1E]). However, the difference between shocked and nonshocked mice persisted across the entire test (P15 shock main effect $P = 0.002$ [Fig. 1D]; P16 shock main effect $P < 0.001$ [Fig. 1E]). Finally, starting at P17 and continuing through adolescence and into adulthood, shocked mice showed stable, high levels of freezing across minutes, with a difference between shocked and nonshocked mice evident throughout the entire test (P17 shock main effect $P < 0.001$ [Fig. 1F]; P30 shock main effect $P < 0.001$ [Fig. 1G]; P60 shock main effect $P < 0.001$ [Fig. 1H]). Therefore, after controlling for age-related differences in spontaneous freezing, we found that the ability of mice to form long-term associations between context and shock emerges at P14 and remains stable in the days thereafter.

Memory specificity

Next, we tested whether the contextual specificity of the memory varied with age. During infancy (P15), adolescence (P30), or adulthood (P60), mice received three foot shocks in a context. Twenty-four hours later, mice were returned to the training context (context A) or placed in one of two different contexts—one that shared several features with the training context (context B) or one that was largely distinct from the training context (context C). We found that, overall, mice discriminated among contexts (context main effect $P < 0.001$) (Fig. 2A–C; Supplemental Table S2), with less freezing in context C compared to in context A ($P < 0.001$) or B ($P < 0.001$). However, there were no differences among ages in the specificity of the context memory.

Memory persistence

We also tested whether the persistence of contextual fear memory depended on age. Infant (P15), adolescent (P30), or adult (P60) mice received three foot shocks in a context, and memory for the context-shock association was tested 1, 7, 14, or 28 d later in separate groups of mice. We observed pronounced age-related differences in freezing across longer training-testing delays (age × delay interaction $P < 0.001$) (Fig. 3A–C; Supplemental Table S3). Consistent with previous reports of infantile amnesia in rats (Campbell and Campbell 1962; Spear 1979), P15 mice showed little or no freezing at training-testing delays longer than 1 d (1 vs. 7 d $P < 0.001$, 1 vs. 14 d $P < 0.001$, 1 vs. 28 d $P < 0.001$) (Fig. 3A). In contrast, P30 and P60 mice showed high levels of freezing for at least 28 d after training (Fig. 3B,C).

The memory loss in infant mice appeared to be extensive, with a complete lack of freezing observed at longer training-testing delays. To assess the severity of this memory loss, we

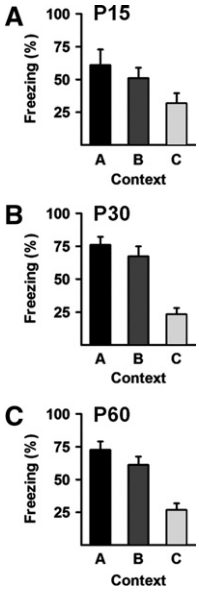

Figure 2. Contextual fear memory specificity. Infant, adolescent, or adult mice received three unsignaled foot shocks in a context. Twenty-four hours later, they were placed in the original training context (context A; P15 $n = 7$, P30 $n = 8$, P60 $n = 6$), a similar context (context B; P15 $n = 8$, P30 $n = 8$, P60 $n = 8$), or a dissimilar context (context C; P15 $n = 8$, P30 $n = 7$, P60 $n = 7$). All ages of mice—P15 (*A*), P30 (*B*), and P60 (*C*)—showed similar discrimination among contexts, with higher levels of freezing in contexts A and B compared to context C.

retrained additional groups of mice upon reaching adulthood to test whether prior memory formation would facilitate relearning of the context-shock association (i.e., savings). At P15, mice either received three foot shocks in a context (trained group) or remained in their home cage (naive group). At P60, mice were returned to the context once a day for 4 d, where they received a single foot shock during the first 3 d. Trained and naive mice showed differences in freezing across days, with mice that were trained during infancy displaying somewhat less freezing than naive mice during the last days (training × day interaction $P = 0.025$) (Fig. 3D; Supplemental Table S4). These results, therefore, provide no clear evidence of a memory savings effect during adulthood.

Developmental delay

Finally, we tested the utility of this approach in detecting early signs of cognitive impairment following early life adversity. During infancy (P13, P14, or P15), control and fetal alcohol-exposed (FAE) mice received three foot shocks in a context, and memory for the context-shock association was tested 24 h later. At P13, within the control group, shocked mice froze more than nonshocked mice during the test ($P < 0.001$) (Fig. 4A; Supplemental Table S5), but within the FAE group, shocked and nonshocked mice showed equivalent levels of freezing (EtOH × shock interaction $P = 0.018$). Similarly, at P14, shocked mice froze more than nonshocked mice within the control group ($P < 0.001$) (Fig. 4D), but there was no significant difference in freezing between shocked and nonshocked mice within the FAE group. At P15, however, shocked mice froze more than nonshocked mice within both control and FAE groups (shock main effect $P < 0.001$) (Fig. 4G). Thus, whereas control mice showed evidence of forming contextual fear memories at P13, such evidence was not observed until P15 for FAE mice, indicating that fetal alcohol exposure delays the onset of this type of memory by ~2 d.

To further examine the effect of fetal alcohol exposure on the development of contextual fear memory formation, we analyzed minute-by-minute changes in freezing during training and testing. During training, control and FAE mice showed similar responses to foot shocks, with shock-induced decreases in freezing at P13 (Fig. 4B,C), no change in freezing at P14 (Fig. 4E,F), and shock-induced increases in freezing at P15 (Fig. 4H,I), suggesting that fetal alcohol exposure did not alter sensitivity to the shock. During testing, however, we observed differences in freezing levels between control and FAE mice. At P13, both control and FAE mice initially froze more if they had previously been shocked (control: shock × minute interaction $P < 0.001$, minute 1 $P < 0.001$, minute 2 $P < 0.001$ [Fig. 4B]; FAE: shock × minute interaction $P = 0.002$, minute 1 $P = 0.020$, minute 2 $P = 0.030$ [Fig. 4C]). At P14, within the control group, shocked mice froze more than nonshocked mice throughout the entire test (shock × minute interaction $P = 0.028$, minute 1 $P < 0.001$, minute 2 $P < 0.001$, minute 3 $P < 0.001$, minute 4 $P = 0.018$, minute 5 $P = 0.029$ [Fig. 4E]), but within the FAE group, there were no differences between shocked and nonshocked mice (Fig. 4F). At P15, control and FAE groups showed equivalent levels of freezing, with shocked mice freezing

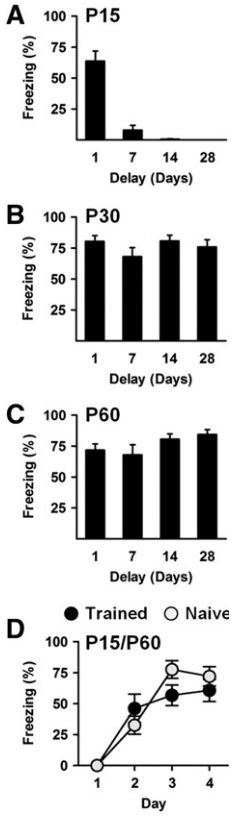

Figure 3. Contextual fear memory persistence. Infant, adolescent, or adult mice received three unsignaled foot shocks in a context and were returned to the context 1 (P15 $n = 8$, P30 $n = 8$, P60 $n = 8$), 7 (P15 $n = 8$, P30 $n = 8$, P60 $n = 7$), 14 (P15 $n = 8$, P30 $n = 8$, P60 $n = 8$), or 28 (P15 $n = 8$, P30 $n = 8$, P60 $n = 8$) days later. P15 mice (*A*) showed little or no freezing at training-testing delays longer than 1 d. In contrast, P30 mice (*B*) and P60 mice (*C*) exhibited high levels of freezing for at least 28 d after training. (*D*) Mice that were either trained (trained group; $n = 8$) or remained in their home cage (naive group; $n = 8$) on P15 underwent 4 d of retraining starting on P60. Mice that were previously trained on P15 showed no evidence of memory savings during retraining.

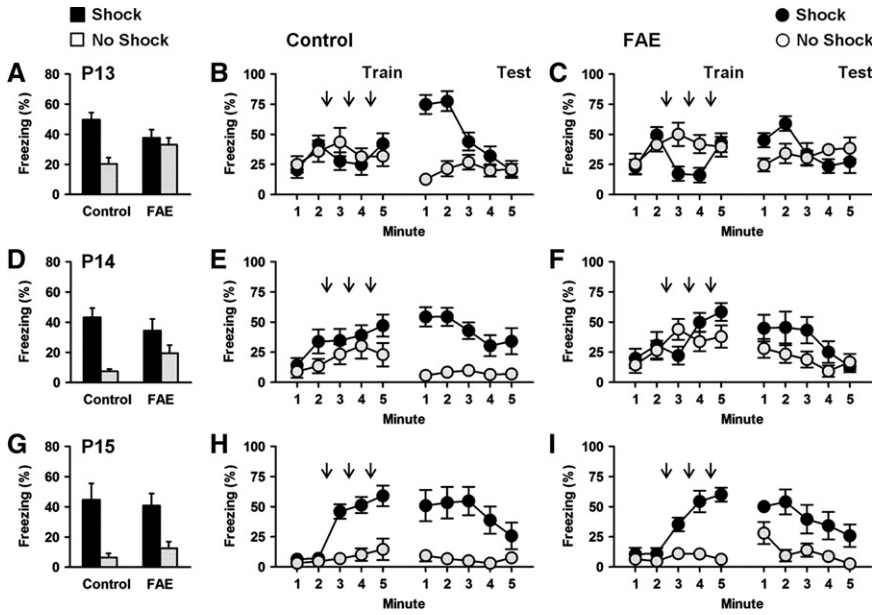

Figure 4. Developmental delay in contextual fear memory formation. Fetal alcohol-exposed (FAE) or control mice were placed in a context, where they either received three unsignaled foot shocks (denoted by a downward arrow [↓]; shock group; FAE: P13 $n = 8$, P14 $n = 9$, P15 $n = 9$; control: P13 $n = 9$, P14 $n = 9$, P15 $n = 9$) or did not receive foot shocks (no shock group; FAE: P13 $n = 9$, P14 $n = 9$, P15 $n = 9$; control: P13 $n = 9$, P14 $n = 9$, P15 $n = 9$). Memory for the context-shock association was tested 24 h after training. At P13 (*A–C*) and P14 (*D–F*), control mice showed clear evidence of forming context-shock associations, but FAE mice showed little such evidence. By P15 (*G–I*), however, both control and FAE mice exhibited evidence of forming contextual fear memories.

more than nonshocked mice during most or all minutes of the test (control: shock × minute interaction $P = 0.006$, minute 1 $P = 0.008$, minute 2 $P = 0.003$, minute 3 $P = 0.001$, minute 4 $P = 0.006$ [Fig. 4H]; FAE: shock main effect $P = 0.009$ [Fig. 4I]).

Discussion

We examined the ontogeny of contextual fear memory formation, specificity, and persistence in mice from infancy to adulthood. After controlling for age-related differences in spontaneous freezing, we found that the ability of mice to form long-term contextual fear memories emerges at 13–14 d of age and remains stable through adolescence and into adulthood. Although the specificity of the memory was similar across ages, infant mice had shorter-lasting memories compared to adolescent and adult mice, indicating that memory persistence continues to develop with age. Finally, we found that ability to associate a shock with a context does not emerge until 15 d of age in mice that were exposed to alcohol during gestation, illustrating the utility of this ontogenetic approach in detecting developmental delays in models of early adversity.

The precise age at which rats begin to form long-term contextual fear memories has been a matter of debate in previous literature. Although some studies report that rats fail to associate a shock with a context until 23–24 d of age (Spear 1979; Rudy 1993; Rudy and Morledge 1994; Raineki et al. 2010; Schiffino et al. 2011), others demonstrate that this type of learning can occur at 17–18 d of age under some training conditions (Brasser and Spear 2004; Esmoris-Arranz et al. 2008; Pisano et al. 2012) or following certain neonatal manipulations (i.e., injection of fibroblast growth factor-2, sensory stimulation) (Woodcock and Richardson 2000; Graham and Richardson 2010; Callaghan and Richardson 2011). Our finding that mice are able to associate a shock with a context as early as 13–14 d of age, however, suggests that brain and behavioral development is accelerated in mice compared to rats, consistent with previous proposals (Pellis and Iwaniuk 2000; Whishaw et al. 2001). Therefore, what is known regarding the ontogenetic timelines of learning and memory in rats may not directly transfer to mice, and this should be taken into account when using mice to model early cognitive and emotional dysfunction.

In adult rodents, hippocampal lesions consistently produce retrograde amnesia for contextual fear memories (Maren et al. 1997; Frankland et al. 1998; Anagnostaras et al. 1999; Wiltgen et al. 2006; Lehmann et al. 2007), providing evidence that this type of memory is usually encoded by the hippocampus. Although it is possible that young rodents could condition to independent elements of the training context through nonhippocampus-dependent processes (Rudy 2009), accumulating evidence suggests that the hippocampus is involved in the formation of contextual fear memories during infancy. Specifically, the developmental onset of contextual fear learning coincides with the onset of training-induced hippocampal immediate early gene expression (Raineki et al. 2010), and lesion or inactivation of the hippocampus blocks the formation of context and contextual fear memories in infant rats (Foster and Burman 2010; Raineki et al. 2010; Schiffino et al. 2011). Therefore, the emerging ability of mice to form context-shock associations at 13–14 d of age may reflect the functional maturation of the hippocampus, which continues to undergo changes in neuron number (Altman and Bayer 1975), dendritic arborization (Rahimi and Claiborne 2007), signaling mechanisms (Paylor et al. 1996), synaptic plasticity (Harris and Teyler 1984), and spatial firing (Langston et al. 2010; Wills et al. 2010; Scott et al. 2011) during the postnatal period.

Furthermore, the emergence of contextual fear memory is likely not solely contingent on hippocampal development but rather the maturation of a broad network of brain regions. Considering that eye opening occurred only 1–2 d prior to the onset of contextual fear learning in mice (i.e., at 12 d of age), the ongoing development of the visual system (Bourne 2010) may also underlie the onset of this type of learning. Furthermore, our observation that spontaneous freezing declined steadily across infancy suggests that the emergence of conditioned fear (as opposed to unconditioned fear) may rely on the maturation of brain regions involved in emotion, such as the amygdala (Gogolla et al. 2009; Chareyron et al. 2012) and prefrontal cortex (van Eden et al. 1990).

In contrast to adolescent and adult mice, which showed long-lasting memory for the context-fear association, infant mice showed rapid forgetting. These findings are consistent with previous studies in rats, which report that infants forget details of the context in which they were shocked more quickly than adults (Anderson and Riccio 2005) and ultimately show complete loss of memory for the context-shock association (i.e., "infantile amnesia") (Campbell and Campbell 1962; Rudy and Morledge 1994; Weber et al. 2006). It is unclear whether this

loss of memory in infants reflects a failure of storage or a failure of retrieval. Fear memory can be restored in infant rats following reminders or pharmacological treatments in the first several days following training (Richardson et al. 1983; Weber et al. 2006; Kim and Richardson 2007), indicating that while memories formed during infancy may be resistant to retrieval, they are still present for at least a short period of time following "forgetting." However, our effort to restore an early fear memory by retraining mice during adulthood yielded no overwhelming evidence of a preserved context-shock association at this age, suggesting that memories lost after infancy may eventually become irrecoverable.

One recent study on the development of contextual fear memory spanning from early adolescence to adulthood found that although mice of all ages could form context-shock associations, the subsequent expression of these memories was suppressed during adolescence, between 29 and 39 d of age (Pattwell et al. 2011). The authors speculated that this temporary suppression of contextual fear memory could serve an adaptive function, allowing adolescent mice to venture out of the nest and explore potentially threatening environments. In line with several previous studies in mice (Hefner and Holmes 2007) and rats (Pugh et al. 1997; Brasser and Spear 2004; Esmoris-Arranz et al. 2008; Murawski and Stanton 2010), however, we found no evidence of memory suppression in adolescents. Instead, we found that levels of conditioned fear were stable across all ages tested, from infancy to adulthood. This suggests that the finding by Pattwell et al. (2011) may not reflect a general developmental phenomenon but rather may be due to a difference in mouse genetic background or a peculiarity in their husbandry or experimental procedures.

The construction of ontogenetic timelines of learning and memory in mice not only provides a developmental approach to studying basic relationships between brain and behavior but also can be used to detect early signs of cognitive impairment in models of childhood-onset disorders. Although many disorders of genetic and/or environmental origin are associated with developmental delays in cognitive function (Cornish et al. 2004; Sagvolden et al. 2005; Riley et al. 2011), most current animal models typically do not assess cognitive function until adulthood or perhaps at one or two isolated developmental stages. For instance, fetal alcohol exposure is consistently found to produce widespread effects on learning and memory in rodents that are present during adolescence and adulthood (Berman and Hannigan 2000; Schneider et al. 2011), but relatively little is known regarding the developmental onset of these effects in younger animals (e.g., Blanchard et al. 1987; Wigal et al. 1988; Kirstein et al. 1997). Our finding that the ability to form context-shock associations is delayed by up to 2 d in fetal alcohol-exposed mice demonstrates that a detailed ontogenetic timeline of contextual fear memory can be used as a template to compare normal vs. abnormal cognitive development. Because this type of information can have important clinical applications (Pine 2009; Bale et al. 2010; Marco et al. 2011), future efforts should be made to construct ontogenetic timelines for other cognitive functions relevant to childhood-onset disorders, such as attention, response inhibition, working memory, and social interaction.

Materials and Methods

Animals

All procedures were approved by the Animal Care Committee at the Hospital for Sick Children. Mice were a cross between C57Bl/6 (paternal) and 129Svev (maternal) strains (Taconic), which were bred in our animal facility and maintained on a 12 h light/dark cycle (lights on at 0700 h). The day of birth was designated P0, with litter sizes ranging from two to 11 pups. Eye opening occurred at P12. After weaning on P21, mice were group-housed (two to five per cage) in transparent plastic cages (31 × 17 × 14 cm). To prevent the influence of litter effects on dependent measures (Abbey and Howard 1972), typically no more than one female and one male from each litter were assigned to a particular experimental group. When more than one same-sex littermate was tested within a group, dependent measures from the littermates were averaged. Roughly equal numbers of males and females were assigned to each group.

Fetal alcohol exposure

The fetal alcohol exposure procedure was adapted from previous studies (Allan et al. 2003; Akers et al. 2011). Briefly, female mice ($n = 10$) were given saccharin-flavored drinking water (0.1% saccharin) to which ethanol (EtOH) was gradually introduced (0% EtOH for 4 d, 2% EtOH for 1 d, 5% for 1 d, 10% for 4 d). Mice continued to receive 10% EtOH during temporary cohabitation with a male breeder and throughout the extent of pregnancy. Starting on the day of birth, the concentration of EtOH was gradually decreased (5% EtOH for 2 d, 2% EtOH for 2 d) and then replaced with regular water. Control mice ($n = 9$) received water containing 0.1% saccharin throughout pregnancy. All mice had free access to regular mouse chow.

Control and EtOH dams consumed an average of 4.89 ± 0.22 and 4.59 ± 0.11 g fluid daily during pregnancy, resulting in an average dose of 0 ± 0 and 14.15 ± 0.43 g/kg/day EtOH, respectively. Although dams continued to drink a reduced amount of EtOH during the first few days after giving birth, the amount of EtOH passed on to pups through lactation is low (~2% of maternal dose) (Mennella 2001), and therefore, its exposure to pups during this period was expected to be minimal. We found no differences between groups in dam body weight at onset of pregnancy (control: 26.8 ± 1.4 g, EtOH: 25.6 ± 0.7 g), weight gained across pregnancy (control: 12.7 ± 1.2 g, EtOH: 13.9 ± 0.6 g), or litter size (control: 6.0 ± 0.6 pups, EtOH: 6.7 ± 0.4 pups). Previous studies have shown that this procedure has no effect on maternal behavior or pup body weight but produces changes in brain volume, levels of neurotrophic factors, and behavior that persist into adulthood (Allan et al. 2003; Caldwell et al. 2008; Akers et al. 2011).

Contextual fear conditioning

Contextual fear conditioning occurred in test chambers (33-cm height, 29.2-cm width, 26.3-cm depth, Coulbourn Instruments, H10-11R-TC). The front, top, and back of the chambers were made of clear acrylic, and the sides were made of modular aluminum. Shock-grid floors were comprised of stainless steel bars (0.4-cm diameter) spaced 0.7 cm apart. Shock delivery was controlled using a shock generator (Coulbourn Instruments, H13-15). Freezing behavior (i.e., cessation of all movement except breathing) was recorded by overhead video cameras and analyzed using automated software (FreezeFrame, Actimetrics). Chambers were cleaned with water between trials.

Memory formation

Separate groups of mice were trained at P13, P14, P15, P16, P17, P30, or P60. Two minutes after being placed in the chambers, mice received three unsignaled foot shocks (2 sec, 0.5 mA, 1 min apart) and were removed 1 min after the final shock. Additional groups of age-matched mice were placed in the chambers for 5 min but did not receive foot shocks; these mice were trained separately from shocked mice so that they did not hear shock-induced vocalizations. Twenty-four hours after training, mice were returned to the chambers for a 5-min test.

Memory specificity

Mice were trained on P15, P30, or P60 as previously described. Twenty-four hours after training, separate groups of mice were

placed in one of three different contexts for a 5-min test (Wang et al. 2009). Context A was the same chamber used during training. Context B was also the same chamber used during training, but a white plastic floor covered the grid bars, a triangular plastic insert was positioned inside the chamber, and black-and-white striped paper covered the front wall. Context C was a white plastic chamber (18 × 18 × 8 in) located in a different room.

Memory persistence

Mice were trained on P15, P30, or P60 as previously described. Separate groups of mice were returned to the chambers 1, 7, 14, or 28 d after training for a 5-min test. Additional groups of mice were either trained or remained in their home cage on P15 and then underwent retraining starting on P60. Retraining occurred across 4 d. On days 1–3, mice received a single foot shock (2 sec, 0.4 mA) 2 min after being returned to the chambers and were removed 1 min after the shock. On day 4, mice were returned to the chambers for 2 min with no foot shock. Freezing was measured before the shock on each retraining day.

Statistical analysis

Data were analyzed using ANOVA. For the memory formation and memory savings experiments, age (P13, P14, P15, P16, P17, P30, P60), shock (shock, no shock), sex (male, female), or training (trained, naive) were between-subject factors, and minute or day was a within-subject factor. Following significant interactions, uncorrected *t*-tests were performed to elucidate the locus of the effects. For the memory specificity and memory persistence experiments, age (P15, P30, P60), sex (male, female), context (A, B, C), or delay (1, 7, 14, 28) were between-subject factors. Following significant interactions or main effects, Tukey's post-hoc tests were performed. For the developmental delay experiment, age (P13, P14, P15), shock (shock, no shock), sex (male, female), and EtOH (control, FAE) were between-subject factors, and minute was a within-subject factor. Planned, uncorrected *t*-tests were performed to test for differences between control and FAE groups at each age. Furthermore, uncorrected *t*-tests were performed following significant interactions to determine the locus of the effects. Complete results of statistical analyses are presented in Supplemental Tables S1–S5.

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