Developmental emergence of persistent memory for contextual and auditory fear in mice

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The ability to generate memories that persist throughout a lifetime (that is, memory persistence) emerges in early development across species. Although it has been shown that persistent fear memories emerge between late infancy and adolescence in mice, it is unclear exactly when this transition takes place, and whether two major fear conditioning tasks, contextual and auditory fear, share the same time line of developmental onset. Here, we compared the ontogeny of remote contextual and auditory fear in C57BL/6J mice across early life. Mice at postnatal day (P)15, 21, 25, 28, and 30 underwent either contextual or auditory fear training and were tested for fear retrieval 1 or 30 d later. We found that mice displayed 30-d memory for context– and tone–fear starting at P25. We did not find sex differences in the ontogeny of either type of fear memory. Furthermore, 30-d contextual fear retrieval led to an increase in the number of c-Fos positive cells in the prelimbic region of the prefrontal cortex only at an age in which the contextual fear memory was successfully retrieved. These data delineate a precise time line for the emergence of persistent contextual and auditory fear memories in mice and suggest that the prelimbic cortex is only recruited for remote memory recall upon the onset of memory persistence.

[Supplemental material is available for this article.]
processing (Takehara et al. 2003; Frankland and Bontempi 2005; Vetere et al. 2011; Wheeler et al. 2013; Bergstrom 2016; Kitamura et al. 2017; Sathiyakumar et al. 2020). Nevertheless, the contribution of brain regions beyond the hippocampus to the remote recall of memories acquired in early life has not been examined.

Here, we explored these premises by establishing the ontogeny of memory persistence in C57BL/6J mice in two well-characterized Pavlovian fear conditioning protocols: contextual (Rudy and Morledge 1994; Anagnostaras et al. 2001; Maren 2001) and auditory (Brown et al. 1951; LeDoux et al. 1990; Phillips and LeDoux 1992; LeDoux 2000; Maren 2001) fear conditioning. We found that mice displayed a similar developmental emergence of persistent memory for both contextual and auditory fear, and further explored a role for the prefrontal cortex in the emergence of remote (30-d) contextual fear memory retrieval. Our findings establish a precise ontogenetic profile for different aspects of averse processing in mice and offer the recruitment of the prefrontal cortex as a potential additional index for the emergence of persistent memory, further advancing our understanding of the neural correlates underlying persistent memory processing in early life.

Results

To investigate the onset of persistent memory for contextual fear, mice of different ages underwent contextual fear conditioning (120-sec baseline period followed by five shocks presented at 60-sec intervals) and were tested following either a 1-d or 30-d delay period. Fear conditioning took place at one of five ages spanning late infancy to adolescence (Spearr 2000): P15, P21, P25, P28, or P30 (Fig. 1A). One day after fear conditioning (1-d retrieval test), all age groups displayed robust freezing to the context, indicating mice in all age groups were able to retain the context–fear association for 24 h (one-way ANOVA, $F_{4,70} = 2.14, P = 0.0852$) (Fig. 1B). To assess at what age C57BL/6J mice are able to generate persistent memories for contextual fear, we trained and tested a separate cohort of animals 30 d after fear conditioning (30-d retrieval test). Both P15 and P21 mice showed minimal freezing 30 d after training, while P25, P28, and P30 mice displayed significantly higher freezing (one-way ANOVA, $F_{4,77} = 12.10, P < 0.0001$; Tukey’s post-hoc test: P15 vs. P21: $P = 0.9997$, P15 vs. P25: $P = 0.0044$, P15 vs. P28: $P = 0.0005$, P15 vs. P30: $P < 0.0001$, P21 vs. P25: $P = 0.0005$, P21 vs. P28: $P = 0.0006$, P21 vs. P30: $P < 0.0001$, P25 vs. P28: $P = 0.9921$, P25 vs. P30: $P = 0.6906$, P28 vs. P30: $P = 0.8857$) (Fig. 1C). Importantly, late weaning at P25 did not rescue the deficits in persistent memory observed in animals

![Figure 1. Ontogeny of persistent memory for contextual fear. C57BL/6J mice were trained in contextual fear at either P15, P21, P25, P28, or P30, then tested for memory retrieval 1 d or 30 d later. Training consisted of a 120-sec acclimation period to the conditioning chamber, followed by five footshocks (0.5 mA, 2 sec) delivered at 60-sec intervals. (A) Schematic of contextual fear conditioning. (B) Percent freezing to the context at the 1-d retrieval test. All age groups exhibited robust freezing. P15, n = 16 (8 males, 8 females); P21, n = 20 (10 males, 10 females); P25, n = 12 (6 males, 6 females); P28, n = 10 (5 males, 5 females); P30, n = 17 (9 males, 8 females). (C) Percent freezing to the context at the 30-d retrieval test. P15 and P21 mice displayed minimal freezing behavior, while P25, P28, and P30 mice showed significantly higher freezing. P15, n = 11 (5 males, 6 females); P21, n = 13 (7 males, 6 females); P25, n = 11 (6 males, 5 females); P28, n = 14 (8 males, 6 females); P30, n = 13 (7 males, 6 females). (D) Schematic of contextual fear and immediate shock conditioning. (E) Percent freezing at the 30-d retrieval test in animals trained at P25. Animals in the contextual fear group exhibited significantly higher freezing compared with the IMS group. Contextual fear, n = 14 (7 males, 7 females); IMS, n = 13 (8 males, 7 females). (F) Percent freezing at the 30-d retrieval test in animals trained at P28. Animals in the contextual fear group exhibited significantly higher freezing compared with the IMS group. Contextual fear, n = 17 (8 males, 9 females); IMS, n = 18 (10 males, 8 females). All data represent mean ± SEM. (*) $P < 0.05$. Female (orange) and male (purple) data points are identified, but no sex differences were found across experiments (see results for details).](image)
ontogenesis of persistent memory for auditory fear. C57BL/6J mice were trained in auditory conditioning protocols, contextual and auditory fear, differ in certain aspects of their circuit requirements (Phillips and LeDoux 2004b; Vetere et al. 2011; Wheeler et al. 2013; DeNardo et al. 2019; Grella et al. 2020). This process. In adult rodents, recruitment of neuronal activity in the prelimbic cortex (PL) has been shown to occur following remote, but not recent, fear memory retrieval (Frankland et al. 2004a; Wheeler et al. 2013; DeNardo et al. 2019; Grella et al. 2020). We therefore looked at neuronal activation in the PL following the 1-d or 30-d contextual fear retrieval in P15 and P30 mice (Fig. 3A). P15 animals, which are unable to successfully retrieve a 30-d old contextual fear memory, showed decreased activation of PL neurons at the remote time point, an effect that is absent in P30 animals. We refined the time line for the developmental emergence of persistent (30-d) memory for contextual fear in C57BL/6J mice, identifying P21–P25 as the interval for when the ability to generate persistent contextual fear memory emerges. We next investigated the

Discussion

We redefined the time line for the developmental emergence of persistent (30-d) memory for contextual fear in C57BL/6J mice, identifying P21–P25 as the interval for when the ability to generate persistent contextual fear memory emerges. We next investigated the
ontogeny of persistent memory for auditory fear, a commonly used behavioral task with partially overlapping features and underlying neural circuitry (Phillips and LeDoux 1992; Maren 2001; Sotres-Bayon et al. 2012; Maren et al. 2013; Herry and Johansen 2014). We found that persistent (30-d) auditory fear memory emerges within a similar time line to that of contextual fear, between P21 and P25. Importantly, late weaning did not rescue the persistent memory deficit seen in P21 animals, suggesting that the effects of same-day weaning on the auditory or contextual fear cohorts were not responsible for their respective absence of persistent memory. These data suggest synchronous emergence of the ability to generate persistent memories for both context– and tone–fear associations in mice. Additionally, we investigated whether the developmental onset of fear memory persistence was accompanied by neural signatures of remote memory recall. We found that P30 animals displayed higher PL activation at the 30-d memory retrieval test compared with the 1-d memory retrieval test, an effect that was absent in P15 mice. These results show that remote (30-d) context re-exposure is unable to trigger fear memory retrieval or PL recruitment in animals trained at P15. These data identify the precise ontogeny of persistent memory for contextual and auditory fear in mice and provide novel insight into the neural correlates of remote memory retrieval across development.

Studies examining the ontogeny of context conditioning in mice are relatively few (Revillo et al. 2015). The onset of persistent (≥28-d) memory for contextual fear in mice has been reported between P15/P17 and P30 (Akers et al. 2012; Guskjolen et al. 2018), a finding that is replicated in our present study. This also suggests a convergence between C57/129 (Akers et al. 2012; Guskjolen et al. 2018) and C57BL/6 mouse strains in the ontogeny of persistent memory for contextual fear. In rats, persistent contextual fear memory has been found to be absent at P30 and earlier ages (Campbell and Campbell 1962; Beane et al. 2002; Weber et al. 2006), with one study showing its emergence at P38 (Campbell and Campbell 1962). Given that the delay intervals between fear training and retrieval tests vary considerably between these rat studies (7 to 42 d) and ours (30 d), and that the requirement for the hippocampus in contextual fear retrieval may differ between these delays (Kim and Fanselow 1992; Anagnostaras et al. 1999), these data may not be directly comparable. Therefore, based on the available studies, we cannot conclusively say that mice display an earlier emergence of persistent memory for contextual fear compared with rats. Furthermore, it is important to

Figure 3. Developmental recruitment of prefrontal cortex following 1-d and 30-d contextual fear retrieval. C57BL/6 mice were trained in contextual fear at either P15 or P30, then re-exposed to the training context 1 d or 30 d later. Ninety-minutes after the retrieval test, mice were perfused for immunohistochemistry against c-Fos. (A) Schematic of contextual fear conditioning and perfusion. (B) Percent c-Fos-positive cells over the total number of Hoechst-stained cells at either 1-d or 30-d retrieval tests in animals trained at P15. No differences were found between retrieval time points. 1-d, n=10 (5 males, 5 females); 30-d, n=10 (3 males, 7 females). (C) Percent c-Fos positive cells over the total number of cells at either the 1-d or 30-d retrieval test in animals trained at P30. c-Fos expression was significantly higher at the 30-d retrieval test compared with the 1-d test. 1 d, n=6 (3 males, 3 females); 30 d, n=6 (3 males, 3 females). All data represent mean±SEM. (*) P<0.05. Female (orange) and male (purple) data points are identified. Scale bar, 50 µm.
note that postnatal age in each species may differ in terms of brain and behavioral development (Clancy et al. 2001). We only found one study looking at remote auditory fear memory during development in rodents, which showed that P23 rats were unable to retain auditory fear memories for 7 d (Li et al. 2012a), consistent with our time line. Although we cannot exclude the possibility that the onset of persistent memory for contextual and auditory fear may diverge within the P21–P25 time window, these data point to a largely consistent ontogenetic time line for persistent memory for contextual and auditory fear in mice and for auditory fear for both mice and rats. We found no evidence of sex differences in the age of onset for persistent contextual or auditory fear conditioning. To our knowledge, sex differences in fear learning during early life have only been found in fear extinction (Park et al. 2017), and had not been examined within the context of the developmental onset of memory persistence in either rats or mice.

The onset of 1-d memory for both contextual and auditory fear in rats has been observed as early as P15–P18 (Mckinzie and Spear 1995; Pugh and Rudy 1996; Beane et al. 2002; Brasser and Spear 2004; Revillo et al. 2015). We found robust 1-d freezing in mice to both context and tone from P15, the earliest age tested, consistent with previous reports (Akers et al. 2012). While several groups vary the number of auditory tone–shock pairings delivered to P15–17 rodents to equate their freezing to that of older animals (Gogolla et al. 2009; Kim et al. 2009; Kim and Richardson 2007, 2008), we and others (Akers et al. 2012; Jablonski et al. 2012; Bath et al. 2016; Guskjolen et al. 2018) found this adjustment was not necessary in contextual fear. These observations point to developmental differences in fear memory processing that are specific to stimulus-association type, even if they do not extend to memory persistence. Finally, we acknowledge that through optimizing training for remote retrieval across ages within each fear category, differences in experimental design limit our ability to directly compare behavioral output for contextual and auditory fear.

Several studies point to hippocampal maturation as a major factor in guiding the onset of fear memory persistence, likely by enabling the long-term retrieval of infant memories. Furthermore, our data show that a correlate of remote memory retrieval, activation of the prefrontal cortex (Takehara et al. 2003; Frankland et al. 2004a; Frankland and Bontempi 2005; Vetere et al. 2011; Wheeler et al. 2013; Kitamura et al. 2017; DeNardo et al. 2019; Grella et al. 2020; Pan et al. 2020; Sathiyakumar et al. 2020), is absent for infant memories. Interestingly, Guskjolen et al. (2018) found that adult retrieval of P17 memories through optogenetic manipulation of hippocampal neuronal ensembles re-instated patterns of neuronal activity in brain regions such as the entorhinal cortex, but not PL. Future work is needed to determine precisely how early life changes in the hippocampus (Akers et al. 2014; Callaghan et al. 2014; Travaglia et al. 2016b, 2018), and/or the prefrontal cortex (Li et al. 2012a,b) may render memories more accessible for retrieval at long delays. Additionally, this group showed that persistent (30-d) spatial memory for the Morris water maze (MWM) is already present in mice at P20 (Guskjolen et al. 2017), considerably earlier than the onset of persistent memory for contextual fear. Combined with our data, this suggests asynchrony in the ontogeny of memory persistence within hippocampus-dependent memory types. While research has shown a role for PL in the remote retrieval of auditory fear memories (Alden-Content et al. 2015), direct assessment of PL engagement in remote auditory fear in early life is necessary to further link the onset of behavior and neural correlates between these two types of fear memory. Overall, these data consolidate a precise time line for the emergence of memory persistence for context–tone–shock associations in mice, a necessary step toward understanding the neural basis of aversive memory encoding and retrieval across the life span.

Materials and Methods

Animals

Male and female C57BL/6 mice (Jackson Laboratory) were bred at the University of Toronto Scarborough and kept on a 12-h light–dark cycle (lights on at 07:00 h) with access to food and water ad libitum. Date of birth was designated postnatal day (P0), with litter sizes ranging from two to nine pups. All litters were randomly divided and evenly distributed (including by sex) between ages (e.g., P15, P21, P25, P28, and P30) and experimental groups (1-d and 30-d retrieval tests), with a minimum of two to three litters per age. Behavioral procedures were performed during the early light phase between 07:00 and 12:00 h. All animal procedures were approved by the Animal Care Committee at the University of Toronto.

Behavioral testing

Contextual fear conditioning

All contextual fear conditioning procedures were conducted in a soundproof stainless steel conditioning chamber (30 cm × 24 cm × 21 cm, Med Associates) containing a removable stainless-steel shock grid floor (context A). Shock grid bars (3.2 mm diameter) were spaced 8.1 mm apart. The grid floor was located above a stainless-steel drop pan, which was cleaned with 70% ethanol between sessions. The front and top of the chamber were made of clear acrylic, and the two sides and back were made of modular aluminum. Mice were fear trained at either P15, P21, P25, P28, or P30. Training consisted of a 120 sec acclimation period to the conditioning chamber, followed by five footshocks (0.5 mA, 2 sec) delivered at 60-sec intervals (Guskjolen et al. 2018). Mice were removed from the chamber 60 sec after the last footshock and transported back into their home cage. Retrieval was conducted either 1 d or 30 d after the initial fear training. During contextual fear retrieval, mice were placed back into the original training chamber (context A) for a duration of 5 min, and freezing behavior was recorded and quantified by an automated motion-sensitive software with a minimum freezing duration of 1 sec for the 5-min duration of the tests (VideoFreeze, Med Associates). For the context-only group (Supplemental Fig. 2), animals underwent the same procedure as described above for contextual fear conditioning, but in the absence of footshocks during training.

Immediate shock

Two age groups, P25 and P28, were used for the immediate shock (IMS) control experiment. Within each age group, mice were split between either a contextual fear conditioning or an IMS group. Mice in the contextual fear conditioning group underwent the same training procedures described above. Mice in the IMS group were placed in context A and received one footshock (0.5 mA, 2 sec) immediately upon entering the chamber. Mice remained in the chamber for an additional 60 sec, and were then removed and transported back into their home cage (Frankland et al. 2004b; Arruda-Carvalho and Clem 2014). Retrieval was conducted 30 d later. During retrieval, mice were placed back into context A
for a duration of 5 min, and freezing behavior was recorded and quantified by an automated motion-sensitive software as described for contextual fear (VideoFreeze, Med Associates).

Auditory fear conditioning

All auditory fear conditioning procedures were conducted in a soundproof stainless steel conditioning chamber (30 cm × 24 cm × 21 cm, Med Associates). Auditory fear training took place in context A, as previously described. Mice were fear trained at either P15, P21, P25, or P30. Following a 120-sec acclimation period to the conditioning chamber, P21, P25, and P30 mice received six presentations of a 10-sec tone (2000 Hz, 80 dB), which coterminated with a footshock (0.6 mA, 1 sec), spaced by 60-sec intervals (adapted from Gogolla et al. 2009). Mice trained at P15 received only one tone-shock pairing, as data from our laboratory established that this equated their 1-d retrieval freezing levels to that of older animals. All mice were removed from the chamber 60 sec after the last tone–shock presentation and transported back into their home cage. Memory retrieval took place either 1 d or 30 d after training in context B. In context B, a white plastic floor covered the shock grids and a white plastic sheet covered the side and back walls in a semicircle. For memory retrieval, following a 120-sec acclimation period, mice were presented with five 10-sec tones (2000 Hz, 8 dB) at 60-sec intervals (adapted from Gogolla et al. 2009). Mice were removed from the chamber 60 sec after the last tone presentation and were transported back into their home cage. Freezing behavior was recorded and quantified by an automated motion-sensitive software with a minimum freezing duration of 1 sec (VideoFreeze, Med Associates). Freezing was measured during baseline (first 120 sec), as well as the five 10-sec tone presentations and averaged across five tones. Due to a COVID19-related laboratory shutdown, five animals had to be tested 4–5 d earlier in the 30-d retrieval test for auditory fear, as it was the last day in which the laboratory was open. Two of these animals were in the P21 group (tested 26 d after training, average percent freezing across tones: 15% and 15.6%) and three in the P25 group (tested 25 d after training, average percent freezing across tones: 0%, 46.5%, and 51.4%). Since this slightly shorter interval (1) remains conceptually consistent with a measurement of persistent memory, (2) is similar or very close to delays commonly used by other groups for remote memory testing (e.g., 28-d delay [Maren et al. 1996; Wang et al. 2009; Goshen et al. 2011; Kwon et al. 2012; Do-Monte et al. 2015; Poulos et al. 2016; Todd et al. 2016] and P21-d delay [Khalaf and Gräff 2019; Grella et al. 2020]), and (3) evidence suggests freezing levels do not significantly change between remote retrieval intervals (Kim and Fanselow 1992; Maren et al. 1996; Do-Monte et al. 2015.), we opted to include them in this data set.

Dedicated cohorts of mice were used in each fear task according to the retention interval.

Delayed weaning

To examine whether same-day weaning influenced the absence of persistent memory in the P21 group, we trained animals in auditory and contextual fear (as described above) at P21, then weaned animals 4 d later at P25. To prevent double litters, male breeders were returned from the breeding cage once the female was pregnant, or in the rare case where pregnancy was not visibly evident, immediately after the birth of a new litter. All animals within one litter were either trained in auditory or contextual fear (i.e., dedicated cohorts), and to minimize litter effects, each experimental condition was comprised of at least two litters.

Immunohistochemistry

Ninety-minutes following the end of the contextual fear retrieval tests for the animals represented in Figure 1, mice were anesthetized and perfused transcardially with 0.01 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA). The brains were extracted and postfixed in 4% PFA overnight. Coronal sections (50 µm) were cut using a vibratome (Leica VT1000s). The primary antibody used was rabbit polyclonal anti-c-Fos (1:5000; Synaptic Systems 226003) in TNB (0.5% Roche blocking reagent in 1× Tris/NaCl buffer). All sections were treated with 1% hydrogen peroxide. Sections were incubated overnight at 4°C with the primary antibody and then for 60 min at room temperature with donkey antirabbit secondary antibody conjugated with horseradish peroxidase (HRP; 1:500; Jackson Immunoresearch 711-036-152) in TNB. Signals were amplified using tyramide signal amplification conjugated with NHS-fluorescein. All sections were treated with Hoechst nuclear dye (1:1000; Thermo Fisher H1399) for 10 min at room temperature. Sections were mounted on slides with Permafluor antifade medium (Fisher Scientific TA-030-FM).

Images and quantification

Images were acquired using an upright epifluorescence microscope (Nikon Eclipse Ni-U). For cell counts in PL, we used one-fifth systematic section sampling fractions covering the entire anteroposterior extent of the PL (Franklin and Paxinos 2007). All cell counts were performed using ImageJ/Fiji (National Institutes of Health). Total Hoechst-stained cell counts were obtained using the “Analyze Particles” tool and optimized to a 3500–65,536 threshold level, 0–infinity square millimeter size, and 0.00–1.00 circularity, as these parameters produced the most accurate total cell counts when compared with our manual counts. c-Fos-positive cell counts were obtained manually using the “Cell Counter” plug-in. Due to variability in total Hoechst-stained cells across groups (one-way ANOVA, F(5,28) = 10.98, P < 0.0001; Tukey’s post-hoc test: P15 1 d vs. P15 30 d: P = 0.0280, P15 1 d vs. P30 1 d: P = 0.0245, P15 1 d vs. P30 30 d: P = 0.6873, P15 30 d vs P30 1 d: P > 0.0001, P15 30 d vs P30 30 d: P = 0.4638, P30 1 d vs P30 30 d: P = 0.0047), we analyzed c-Fos expression as a percentage of the total number of cells sampled, calculated by dividing the number of c-fos+ cells by the total number of Hoechst-stained cells (adapted from Grella et al. 2020).

Statistical analysis

Data are presented as mean ± SEM. All statistical analyses were obtained using Graphpad Prism software. To compare freezing behavior of animals across age groups within the 1-d or the 30-d groups, one-way ANOVA tests were performed, followed by Tukey’s post-hoc multiple comparisons tests. For the immediate shock experiment, an unpaired two-tailed t-test was conducted for each age group of interest. To determine any differences in percent c-Fos at 1-d versus 30-d retrieval time points, an unpaired two-tailed t-test was performed for each age group of interest. Potential sex differences were first assessed using a two-way ANOVA. Since we found no significant effects of sex on 1-d or 30-d memory retrieval for contextual or auditory fear, data were collapsed across these variables for subsequent analyses.

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