Research

Enhanced generalization of auditory conditioned fear in juvenile mice

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Increased emotionality is a characteristic of human adolescence, but its animal models are limited. Here we report that generalization of auditory conditioned fear between a conditional stimulus (CS+) and a novel auditory stimulus is stronger in 4–5-wk-old mice (juveniles) than in their 9–10-wk-old counterparts (adults), whereas nonassociative sensitization induced by foot shock (US) and the ability to discriminate CS+ from an explicitly unpaired stimulus (CS–) are not dependent on age. These results suggest that aversive associations are less precise in juvenile mice and can more easily produce conditional responses to stimuli different from CS+. Yet, through the explicit unpairing of CS– from US during training, juveniles are able to overcome this greater fear generalization and learn that CS– is not associated with foot shock.

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Adolescence is a critical period for emotional development in humans. It is characterized by robust reorganization of the brain (Steinberg 2005) and the possible onset of several psychiatric disorders (Cunningham et al. 2002) that are thought to be triggered by developmental changes (Benes 1997; Huttenlocher 1984). Compared with adults, human adolescents exhibit higher instability and intensity in their emotional responses (Larson et al. 1980; Diener et al. 1985), which are often associated with maladaptive actions such as higher risk-taking and novelty-seeking behaviors. Yet, robust animal models reflecting the distinct emotionality in adolescents are rather limited (Spear 2000).

Emotional behaviors in rodents can be assessed by Pavlovian fear conditioning, a paradigm where animals learn to associate a neutral conditional stimulus (CS+) with an aversive unconditional stimulus (US). In this paradigm, maladaptive behaviors can appear as enhanced or diminished freezing to CS+ or as excessive fear generalization in the form of additional freezing to stimuli different from CS+. In the real world, where danger-predicting cues are similar but rarely identical to those associated with past aversive experiences, fear generalization serves as a necessary adaptation to develop defensive responses to potential predictors of danger (Gonzalez et al. 2003). Usually, the expression of defensive behaviors is restricted to situations of actual danger, because generalization occurs such that the more a stimulus resembles the original CS+, the greater the conditional response (Honig and Urcuioli 1981). However, excessive fear generalization can induce defensive behaviors to stimuli that do not predict danger, and therefore hinder an animal's ability to compete for resources.

Early juvenile rodents have been extensively studied in the ontogeny of fear conditioning (Rudy and Cheate 1977; Hunt et al. 1994; Hunt 1999; Richardson and Fan 2002), but no work has been performed on late juveniles, except for a study reporting enhanced fear conditioning in 4–5-wk-old C57BL/6 mice, which are considered to be a match for adolescent humans (Hefner and Holmes 2007). For fear generalization, however, no comparative

study has been done between late juvenile and adult mice. Here we investigate how generalization of auditory fear conditioning evolves during the transition from the late juvenile period to adulthood in mice and propose that enhanced fear generalization in juvenile mice can serve as a model for the heightened emotionality in adolescent humans.

Results

Fear generalization in juvenile and adult mice

In the auditory fear conditioning paradigm, fear generalization can develop not only to an auditory cue different from CS+, but also to a novel testing context. To determine the optimal conditions for measuring fear generalization to auditory cues, we first compared fear learning between juvenile (4–5-wk-old) and adult (9–10-wk-old) animals using the same training protocol: five pairings between CS+ (continuous 8-kHz tone) and US (0.5 mA electrical shock, 0.5 sec duration; Fig. 1A). Twenty-four hours after training, when placed in a novel testing context, juveniles froze more than adults (Pre-CS in Testing; juvenile: $26.6 \pm 7.9\%$, $n = 12$; adult: $8.1 \pm 3.1\%$, $n = 12$, $P = 0.04$; Fig. 1B), showing higher levels of contextual fear generalization. During subsequent presentation of CS+, juvenile mice also had a tendency to freeze more (CS in Testing; juvenile: $76.7 \pm 6.0\%$; adult: $66.6 \pm 2.9\%$, $P = 0.14$), which is consistent with the report of more robust auditory fear learning in juvenile mice (Hefner and Holmes 2007). Since contextual fear generalization can be attenuated by decreasing the number or intensity of US presentations (Laxmi et al. 2003; Baldi et al. 2004), in subsequent experiments we trained juvenile mice with four instead of five CS–US pairings.

To examine fear generalization to an unfamiliar auditory cue, we used a nondifferential conditioning protocol. Juvenile and adult mice were each divided into two groups: one of which (matched group) was trained using a patterned tone (pips), while the other (unmatched group) was trained using a continuous tone as CS (Fig. 2A). The animals were tested 24 h later in a novel context using the pips. The two groups of adult mice had similar levels of freezing to a training context (Pre-CS in Training; adult matched: $6.1 \pm 2.6\%$, $n = 8$; adult unmatched: $7.7 \pm 3.8\%$, $n = 8$, $P = 0.73$), to CS before shock (CS in Training; adult matched: $6.4 \pm$

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Figure 1. Higher fear generalization to context in juvenile mice following training with five CS–US pairings. (A) Experimental scheme. (B) Percent freezing time in a training context before CS onset (Pre-CS in Training), during the first CS (CS in Training), in a testing context 24 h after training (Pre-CS in Testing), and during CS presentation (CS in Testing). (Open bars) Juveniles; (gray bars) adults; (*) $P < 0.05$.

4.5%; adult unmatched: $9.1 \pm 5.0\%$, $P = 0.70$), and to a novel testing context (Pre-Pips in Testing; adult matched: $3.7 \pm 2.7\%$; adult unmatched: $3.2 \pm 2.2\%$; $P = 0.87$). Yet, the two groups of adult mice showed different levels of freezing to the pips; mice trained with the continuous tone froze less than mice trained with the pips (Pips in Testing; adult matched: $68.6 \pm 8.7\%$; adult unmatched: $40.8 \pm 7.3\%$; $P = 0.03$; Fig. 2C).

In contrast to the adults, the two groups of juvenile mice did not differ in their freezing to the pips during testing (Pips in Testing; juvenile matched: $53.7 \pm 6.9\%$, $n = 8$; juvenile unmatched: $49.8 \pm 9.8\%$, $n = 8$, $P = 0.75$; Fig. 2B). Neither did they differ in the level of freezing to a training context (Pre-CS in Training; juvenile matched: $2.7 \pm 1.1\%$; juvenile unmatched: $4.8 \pm 2.1\%$, $P = 0.39$), to CS before shock (CS in Training; juvenile matched: $2.1 \pm 1.4\%$; juvenile unmatched: $1.3 \pm 1.3\%$, $P = 0.72$), nor to a novel testing context 24 h after training (Pre-Pips in Testing; juvenile matched: $0.4 \pm 0.4\%$; juvenile unmatched: $4.4 \pm 2.7\%$, $P = 0.17$). Moreover, fear generalization to context no longer differed between juvenile and adult animals (Pre-Pips in Testing; juvenile: $2.4 \pm 1.4\%$, $n = 16$; adults: $3.4 \pm 1.7\%$, $n = 16$, $P = 0.64$), most likely because of the reduced number of CS–US pairing used for conditioning of the juveniles.

This similar freezing to the pips between the juvenile groups did not appear to result from a decrease in the number of CS–US pairings from five to four, because when adult mice were trained with four CS–US pairings, the pip-trained adult mice still exhibited more freezing to the pips than adult mice trained with the continuous tone (Pips in Testing; adult matched: $64.9 \pm 5.2\%$; adult unmatched: $31.7 \pm 8.7\%$; $P = 0.004$; Supplemental Fig. 1). These results suggest that juvenile mice generalized fear more than adult mice, and that this generalization occurred along the pattern dimension of the auditory signal since both CSs, the continuous tone and pips, were of the same frequency.

In addition, when we used the continuous tone instead of the pips as the testing CS and repeated the fear generalization test on separate groups of mice, the freezing in both age groups did not differ between animals trained with the tone or pips (Tone in Testing; adult matched: $59.4 \pm 5.0\%$; adult unmatched: $61.5 \pm 7.8\%$; $P = 0.83$; juvenile matched: $60.7 \pm 5.4\%$; juvenile unmatched: $53.8 \pm 10.5\%$; $P = 0.57$; Supplemental Fig. 2). This result indicates that in juvenile mice fear generalization from the auditory cues is symmetrical, i.e., not affected by the reversal of the training and testing stimuli; whereas adult mice generalize from continuous tone to pips more than from pips to continuous tone.

We next examined fear generalization along the dimension of frequency by using CSs of different pitch. We trained juvenile and adult mice with continuous tones of either 8 or 12 kHz and tested them 24 h later, such that half of the mice (matched group) were tested with the tone used in the training (matched tone), and the other half (unmatched group) were tested with the tone not used in the training (unmatched tone). In adults the matched group froze more to the testing tone than the unmatched group (adult matched: $59.3 \pm 6.0\%$, $n = 12$; adult unmatched: $43.7 \pm 3.4\%$, $n = 12$, $P = 0.034$), whereas in juveniles the matched and unmatched groups did not differ in their freezing (juvenile matched: $54.6 \pm 6.5\%$, $n = 12$; juvenile unmatched: $61.9 \pm 6.0\%$, $n = 12$, $P = 0.422$; Supplemental Fig. 3). These data indicate

Figure 2. Auditory fear generalization from continuous to patterned tone is higher in juvenile mice than in adults. (A) Experimental scheme. (B, C) Percent freezing time in juvenile (B) and adult (C) mice trained with pips (filled bars: matched group) or a continuous tone (open bars: unmatched group), in a training context before CS onset (Pre-CS in Training), during the first CS (CS in Training), in a testing context 24 h after training (Pre-Pips in Testing), and during the pips presentation (Pips in Testing). (*) $P < 0.05$.

that juvenile mice exhibit stronger fear generalization between auditory stimuli not only along the pattern dimension, but also along the frequency dimension.

As an alternative explanation to higher fear generalization, juvenile mice may simply become more sensitized by electrical shocks and therefore respond to any auditory signal with greater freezing (Kamprath and Wotjak 2004). This interpretation would be likely if juveniles were more sensitive to pain.

US-induced sensitization to a novel auditory stimulus does not differ between juveniles and adults

To test pain sensitivity in mice of both age groups, we used a hot plate test. When placed on a heated plate, juvenile mice showed shorter latency to lick rear paws (juvenile: 14.9 ± 1.0 sec, $n = 22$; adult: 17.6 ± 1.0 sec, $n = 23$, $P = 0.03$; Fig. 3A), which indicates a higher sensitivity to pain.

To compare sensitization by foot shocks to a novel auditory cue between juveniles and adults, mice were exposed to foot shocks (four for juveniles and five for adults) in the absence of auditory cues and tested 24 h later in a novel context in the presence of the pips (Fig. 3B). In a training context before receiving shocks, mice of both ages froze similarly (Pre-Shock in Training; juvenile: $2.2 \pm 2.2\%$, $n = 12$; adult: $2.0 \pm 1.0\%$, $n = 12$, $P = 0.93$; Fig. 3C). When presented with the pips, a novel auditory cue, animals of both ages significantly increased freezing in comparison to that before receiving shocks (mean difference in freezing; juvenile: 19.3% , $P = 0.002$; adult: 20.2% , $P = 0.008$, paired t -test), but there was no difference in freezing between the ages (Pips in Training; juvenile: $21.5 \pm 4.4\%$; adult: $22.2 \pm 6.0\%$, $P = 0.92$) indicating that, under our training conditions, the level of US-induced sensitization to a novel auditory cue did not differ between juvenile and adult animals, notwithstanding their different sensitivities to pain.

Pre-exposure to auditory cues does not eliminate fear generalization in juvenile mice

Stimulus novelty increases fear generalization (Best and Batson 1977), whereas stimulus familiarity improves stimulus discrimination (Honey and Hall 1989). If juvenile mice generalized fear due to higher reactivity to the novelty component of auditory stimuli, familiarization with the stimuli before training should improve stimulus discrimination. To this end, we pre-exposed juvenile mice to the continuous tone and pips during 2 d, and repeated the nondifferential conditioning test (Fig. 4A). Despite this pre-exposure, matched and unmatched groups of juvenile animals trained with either of the auditory cues still showed identical levels of freezing when presented with the pips (Pips in Training; matched: $35.3 \pm 8.9\%$, $n = 8$; unmatched: $36.8 \pm 8.7\%$, $n = 8$, $P =$

0.90; Fig. 4B). Yet, the pre-exposed mice froze to the pips less than the non-pre-exposed animals (pre-exposed: $36.1 \pm 6.0\%$, $n = 16$; non-pre-exposed: $51.8 \pm 5.8\%$, $n = 16$, $P = 0.035$, upper tail t -test), as expected from the effect of latent inhibition (Lubow 1989). Thus, we could not suppress fear generalization in juvenile mice by excluding the novelty component of the auditory stimuli.

Juvenile mice do not generalize conditioned fear to an explicitly unpaired cue

We next tested whether juvenile animals could overcome fear generalization by being exposed to a cue presented as explicitly unpaired from foot shock during training. To this end we used a differential conditioning protocol where two auditory cues were presented, but only one cue (CS+) was paired with US (Fig. 5A). When tested using the pips 24 h after training, in both age groups animals trained with the pips as CS+ showed more freezing than animals trained with the continuous tone as CS+ (Fig. 5B,C; Pips in Training; juvenile matched: $70.2 \pm 4.0\%$, $n = 12$; juvenile unmatched: $46.2 \pm 6.0\%$, $n = 12$, $P = 0.003$; adult matched: $67.9 \pm 5.5\%$, $n = 16$; adult unmatched: $50.8 \pm 6.4\%$, $n = 16$, $P = 0.05$) but did not differ in freezing to a training context (Pre-CS in Training; juvenile matched: $5.4 \pm 2.7\%$; juvenile unmatched: $2.2 \pm 1.6\%$, $P = 0.33$; adults matched: $0.7 \pm 0.7\%$; adult unmatched: $2.5 \pm 1.9\%$, $P = 0.35$), to CS before shock (CS in Training; juvenile matched: $6.3 \pm 2.6\%$; juvenile unmatched: $5.6 \pm 2.9\%$, $P = 0.85$; adult matched: $5.2 \pm 4.4\%$; adult unmatched: $2.2 \pm 1.8\%$, $P = 0.53$), nor to a novel testing context 24 h after training (Pre-Pips; juvenile matched: $8.9 \pm 4.5\%$; juvenile unmatched: $15.0 \pm 8.3\%$, $P = 0.52$; adult matched: $8.1 \pm 2.7\%$; adult unmatched: $8.2 \pm 2.6\%$, $P = 0.98$). Thus, juvenile animals were able to distinguish between the two auditory cues and did not generalize fear more than adults to a cue explicitly unpaired from US during training.

Discussion

In the present work we tested conditioned fear generalization between different auditory signals in juvenile and adult mice. As in previous studies (Tang et al. 2003; Senkov et al. 2006), adult mice efficiently discriminated between continuous and patterned tones of the same frequency: When tested with the pips, animals trained with the continuous tone froze less than animals trained with the pips. This difference in freezing did not result from lower salience of the continuous tone as CS when compared with the pips, because adult mice trained and tested with the tone (Fig. 1) showed similar levels of freezing with the adult mice trained and tested with the pips (Fig. 2C) (tone: $66.6 \pm 2.9\%$, $n = 12$; pips: $68.6 \pm 8.7\%$, $n = 8$, $P = 0.80$). Notably, in contrast to adult mice trained with the continuous tone and tested with pips, adult mice completely generalized fear from the training pips to the testing continuous tone. Thus, adult mice exhibited asymmetrical generalization along the pattern dimension between two auditory CSs (animals trained with CS1 generalize to CS2 more than animals trained with CS2 generalize to CS1), which has been described previously (Bang et al. 2008). Meanwhile, consistent with a study in rats on fear generalization along the frequency dimension (Armony et al. 1997), adult mice in this study discriminated between two continuous tones of different frequencies; yet they did not exhibit a tendency for asymmetrical fear generalization between these two tones (data not shown).

In contrast to adults, two groups of juvenile mice trained with either contin-

Figure 3. Higher pain sensitivity does not result in greater sensitization to pips in juveniles. (A) Pain sensitivity measured in the hot plate test and expressed as latency to lick rear paws. (B,C) Sensitization to pips following exposure to electrical shocks. (B) Experimental scheme. (C) Percent freezing before the first shock in a training context (Pre-Shock in Training), in a testing context 24 h after training (Pre-Pips, Testing), and during the pips (Pips, Testing). (Open bars) juveniles; (gray bars) adults; (*) $P < 0.05$.

Figure 4. Pre-exposure to auditory cues for auditory cues does not prevent fear generalization in juveniles. (A) Experimental scheme. (B) Percent freezing in juvenile mice pre-exposed to a continuous tone and pips, then trained with either pips (filled bars: matched group) or a continuous tone (open bars: unmatched group) and tested with the pips. Abbreviations as in Figure 2.

uous tone or pips showed the same level of freezing to both stimuli. Moreover, juveniles showed the same level of freezing to continuous tone, regardless of whether it was of the same or different frequency from the tone used during training. These results suggest that juvenile mice generalize fear more than adult animals do, and this generalization can occur along both the pattern and the frequency dimension of CS.

Alternatively, or in addition to differences in fear generalization, juveniles may develop stronger nonassociative sensitization to an auditory cue following exposure to electrical shocks. The latter possibility has been ruled out by identical levels of non-associative freezing to pips following exposure of animals to electrical shocks in the absence of auditory cues.

The next question was whether enhanced fear generalization is an unavoidable phenomenon accompanying aversive associations in juveniles, or whether juveniles can overcome this trait under training conditions that allow better learning of the distinction between the two cues. To this end, we either used a discriminative procedure to present juvenile mice with both cues during training or familiarized animals with the cues prior to training. Training animals with two cues, one paired and another one explicitly unpaired with US, enabled mice to readily discriminate between the cues during testing. However, the pre-exposure to both cues before training did not reduce fear generalization in juvenile animals. These findings indicated that juvenile mice are not deficient in distinguishing between the two auditory cues, and that the discriminative training yields better stimulus control in juveniles. In contrast, adult mice did not improve their discrimination between CS+ and CS− following the discriminative

training (cf. Figs. 2C and 5C). One possible reason for such disparity is an inherent lack of attention in mice to stimuli that do not have reinforcing contingencies (Cho et al. 1998; Rotenberg et al. 2000; Kentros et al. 2004), such as the CS− in this study. Why then do juvenile mice show better stimulus control after the explicit discriminative training? A previous study in rats found that the duration of attention to a novel last-ing auditory stimulus is longer in juveniles than in adults (Hayne et al. 1992). This finding suggests that juveniles pay more attention to the CS− than adults, have better safety learning (Rogan et al. 2005), and as a result, develop better stimulus control following the discriminative procedure. However, proving this hypothesis requires further investigation.

Synaptic plasticity in the thalamic and cortical inputs to the lateral amygdala (LA) underlies auditory fear conditioning (McKernan and Shinnick-Gallagher 1997; Tsvetkov et al. 2002). Augmentation of plasticity in these inputs is thought to cause fear generalization based on the observations in GABA_B receptor knockout mice (Shaban et al. 2006) and in rats expressing a dominant active form of CREB in thalamic neurons (Han et al. 2008). The present study's finding of stronger fear generalization in juveniles, along with the discovery of a developmental decline of synaptic plasticity in the thalamic input to LA during the transition from the late juvenile period to adulthood (B-X. Pan, W. Ito, and A. Morozov, in prep.), is consistent with the studies mentioned above and support the idea that heightened plasticity in amygdala inputs might be one mechanism responsible for fear generalization. However, additional investigation is required to establish a causal link between the two phenomena.

Yet, stronger fear generalization in juveniles occurs not only between auditory cues but also between contexts (Fig. 1B). This suggests that late developmental changes may also take place in areas outside of the amygdala, such as the hippocampus, and contribute to the difference in contextual fear generalization between juveniles and adults.

Heightened generalization of fear can cause excessive defensive responses to stimuli that do not predict danger, thereby suppressing an animal's ability to compete for resources. At the same time, it may also promote survival in juvenile mice, which are less experienced, more vulnerable, and thus more apt to use broader safety margins while detecting threat. Yet, our finding that a discriminative training procedure, where one auditory stimulus was explicitly unpaired from US, prevented fear generalization regardless of age (Fig. 5B,C) indicates that effective safety learning may serve as a means to counter enhanced juvenile fear generalization.

Materials and Methods

Animals

All experiments were performed on 4–5 and 9–10-wk-old B6/129 F1-hybrid male mice (B6129F1; Taconic Farms) and approved by NIMH Animal Care and Use Committee.

Figure 5. Juvenile animals avoid fear generalization to a cue explicitly unpaired from US. (A) Experimental scheme. (B,C) Percent freezing of juvenile (B) and adult (C) animals trained using a discriminative training paradigm, where either pips (filled bars: matched group) or a continuous tone (open bars: unmatched group) were served as CS+ and tested 24 h later with the pips. Abbreviations as in Figure 2, (**) $P < 0.01$; (*) $P < 0.05$.

Behavior

Fear conditioning

All naive mice were handled by experimenters for 2 min per day for two consecutive days prior to experiments.

Auditory fear conditioning was performed generally as described (Herry and Garcia 2002). Mice were placed in a conditioning chamber (Med Associates) for 2 min, and then five pairings between CS (a continuous 85-dB tone; 8 kHz, 30 sec) and US (a mild electrical shock; 0.5 mA, 0.5 sec) were presented in random intervals (60–180 sec). CS coterminated with US in each pairing. Thirty seconds after the last shock, mice were returned to their home cages. Twenty-four hours after training, mice were placed in a different context for 3 min, and CS was presented during the last 2 min of the session. Freezing, defined as a complete lack of movement except breathing, was quantified by a video-based system (FreezeFrame; Actimetrics) during the training session before onset of the first tone (Pre-CS in Training) and during the first tone (CS in Training), during the testing session before onset of a tone (Pre-CS in Testing) and during the tone (CS in Testing). Percent freezing time was calculated for each period.

To assess fear generalization from nondifferential conditioning, the above procedure was used with the following modifications. Juvenile mice were trained with four, whereas adults were trained with either four or five CS–US pairings. CS was 85 dB continuous tones of either 8 or 12 kHz, or a patterned 85-dB tone (2 pips per second) of 8 kHz. Separate groups of mice were tested only once with one of the three CSs each.

For differential conditioning (Bang et al. 2008), mice were trained with alternating continuous and patterned 8 kHz tones

(four times each tone for juveniles and five times each tone for adults) presented at variable intervals (30–90 sec) in such a way that one of the two tones (CS+) coterminated with US, but the other one (CS–) did not.

In experiments with CS pre-exposure, mice were presented with the two tones during two consecutive days before training in the same way as for the differential conditioning, except US was omitted. Training was performed in the same manner as for the nondifferential conditioning.

For measuring sensitization by electrical shocks to a novel auditory cue, mice received electrical shocks (four times for juveniles, five for adults) without receiving an auditory cue and tested 24 h later with the patterned tone in a novel context. Data are presented as mean $\pm$ SEM. Statistical comparisons were made using the two-tail t -test, unless specified otherwise.

Hot plate test

One hour before testing, mice were habituated to an apparatus (Analgesia meter, Coulbourn Instruments) for 3 min with the heater off. For measuring pain sensitivity, mice were placed on a hot plate heated to 55°C and removed from the plate immediately after they began to lick their rear paws, which was no later than 30 sec after being placed on the plate. The latency to lick rear paws was recorded. Data are presented as mean $\pm$ SEM. Statistical comparisons were made using an upper tail t -test.

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