Representation of Fear of Heights by Basolateral Amygdala Neurons

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Fear of heights is evolutionarily important for survival, yet it is unclear how and which brain regions process such height threats. Given the importance of the basolateral amygdala (BLA) in mediating both learned and innate fear, we investigated how BLA neurons may respond to high-place exposure in freely behaving male mice. We found that a discrete set of BLA neurons exhibited robust firing increases when the mouse was either exploring or placed on a high place, accompanied by increased heart rate and freezing. Importantly, these high-place fear neurons were only activated under height threats, but not looming, acoustic startle, predatory odor, or mild anxiogenic conditions. Furthermore, after a fear-conditioning procedure, these high-place fear neurons developed conditioned responses to the context, but not the cue, indicating a convergence in processing of dangerous/risky contextual information. Our results provide insights into the neuronal representation of the fear of heights and may have implications for the treatment of excessive fear disorders.

Key words: anxiety; basolateral amygdala; fear conditioning; fear of heights; innate fear

Significance Statement

Fear can be innate or learned, as innate fear does not require any associative learning or experiences. Previous research mainly focused on studying the neural mechanism of learned fear, often using an associative conditioning procedure such as pairing a tone with a footshock. Only recently scientists started to investigate the neural circuits of innate fear, including the fear of predator odors and looming visual threats; however, how the brain processes the innate fear of heights is unclear. Here we provide direct evidence that the basolateral amygdala (BLA) is involved in representing the fear of heights. A subpopulation of BLA neurons exhibits a selective response to height and contextual threats, but not to other fear-related sensory or anxiogenic stimuli.

Introduction

Fear of heights is one of the most fundamental survival responses, often accompanied by physiological and behavioral changes such as increase in heart rate (HR), freezing, and avoidance. It is known that the ability to detect and respond to height threats is evolutionarily conserved across a wide range of species (Gibson and Walk, 1960; Poulton et al., 1998). Importantly, fear of heights appears to be innate because associative learning is not

necessary, as seen by the avoidance of a “visual cliff” in newborn animals and human infants (Gibson and Walk, 1960; Scarr and Salapatek, 1970; Menzies and Clarke, 1995). This likely maximizes survival rates against height-related hazards, such as falling from a cliff. On the other hand, irrational or excessive fear of heights can lead to acrophobia, which interferes with normal daily activity (Depla et al., 2008; Arroll et al., 2017). It is estimated that the life-time prevalence of visual height intolerance is as high as 28% in the general population and about half of those who are susceptible report an impact on quality of life (Huppert et al., 2013). Therefore, understanding how the fear of heights is processed in the brain could provide insight into a better understanding of such maladaptive behaviors, as well as clinical implications for treatment. Currently, the neural circuitry that processes height threats remains unknown.

The amygdala, specifically the basolateral amygdala (BLA), receives multimodal sensory inputs from both cortical and subcortical regions and is well known for its role in mediating conditioned fear (LeDoux, 2000; Maren, 2001; Herry et al., 2008). Growing evidence also suggests that the amygdala is critical for

Received Feb. 28, 2020; revised Oct. 9, 2020; accepted Oct. 17, 2020.

Author contributions: J.L., L.L., and D.V.W. designed research; J.L. and D.V.W. performed research; J.L. and D.V.W. analyzed data; J.L. and D.V.W. wrote the paper.

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This research was supported by National Institute of Mental Health/National Institutes of Health (Grant R01-MH-119102 to D.V.W.) and the National Natural Science Foundation of China (Grant 31661143038 to L.L.). We thank Ashley Opalka and Candace Rizzi-Wise for the editing of the manuscript.

The authors declare no competing interests.

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<https://doi.org/10.1523/JNEUROSCI.0483-20.2020>

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Figure 1. Cup handling evokes BLA activity and heart rate increase. **A**, Top, Schematic drawing of unilateral multitetrode (8 or 16) recording in the BLA. Bottom, A representative coronal section and schematics showing the recording sites in the BLA. Red squares indicate final recording sites of individual mice ($n = 16$). Scale bar, 1 mm. **B**, Left, Illustration of cup handling. Right, Activity of one representative BLA neuron (top) and instant heart rates (bottom) recorded simultaneously on cup handling. The durations of the three handlings were 38, 50, and 34 s, respectively.

innate fear (Blanchard and Blanchard, 1972; Davis, 1997; Shumyatsky et al., 2005). Specifically, recent studies revealed that the thalamus-to-BLA and anterior cingulate cortex-to-BLA pathways are important for innate fear responses to overhead looming stimuli and predator odor, respectively (Wei et al., 2015; Jhang et al., 2018; Salay et al., 2018). These findings raise the possibility that the BLA also processes the innate fear of heights. In the present study, we investigated whether and how the BLA neurons represented fear of heights, using multichannel *in vivo* tetrode recording. We also examined a possible relationship between innate and conditioned (learned) fears processed by the same BLA neural population.

Materials and Methods

Animals

All procedures were performed in accordance with the National Institutes of Health *Guide to the Care and Use of Experimental Animals* and the Animals Act, 2006 (People's Republic of China); the animal protocol was approved by the *Institutional Animal Care and Use Committee* at Drexel University. Male C57BL/6 mice were used in all experiments (~12 weeks old at the time of surgery; we decided to use only male mice to avoid any potential interference in physiological and behavioral responses by odor from the opposite sex, though we do not expect to see a difference between male and female mice). A total of 35 mice received tetrode implantation for *in vivo* recording [5 mice also received implantations for electrocardiogram (EKG) recording]; our spike analysis was based on 16 of these mice in which we recorded at least one high-place fear neuron (Fig. 1A). After surgery, mice were singly housed in home cages (45 × 24 × 20 cm) on a 12 h light/dark cycle with *ad libitum* access to food and water. The cup-handling procedure (Hurst and West, 2010) was performed twice a day (~5 min each session) for at least 3 d before the initiation of any test to minimize potential stress from the experimenter.

Surgery

The tetrode implantation surgery is similar to that described previously (Wang et al., 2015; Wang and Ikemoto, 2016). Briefly, mice were given an intraperitoneal injection of ketamine (100 mg/kg; Vedco)/xylazine (10 mg/kg; Akorn) mixture before the surgery. Among the 16 mice (Fig. 1A), 4 were implanted with a bundle of 16 tetrodes, and the other 12

mice were implanted with a bundle of 8 tetrodes. The stereotaxic coordinates used for targeting the BLA (right hemisphere; we decided to target only the right hemisphere mainly because of the convenience of our surgery setup, though we do not expect to see a difference between the two hemispheres) were as follows: 1.4 mm posterior to bregma, 3.3 mm lateral to midline, and −3.8 mm ventral to the brain surface. The electrode bundle was coupled with a microdrive and, thus, can be advanced into deeper regions over several days in small daily increments (Wang et al., 2011, 2015). The EKG lead implantation was similar to that described previously (Liu et al., 2013, 2014). Briefly, a pair of insulated wires was penetrated subcutaneously from the back of the neck to the chest in the lead II configuration (McCauley and Wehrens, 2010): the positive lead was placed in the left abdomen below the heart, while the negative lead was placed in the upper right chest. The mice were allowed to recover for at least 5 d before any test was initiated.

Tetrode and EKG recording

Each tetrode consisted of four wires twisted together (Fe-Ni-Cr/Stablohm 675 wire, 13 μ m diameter; or 90% platinum/10% iridium, 18 μ m diameter; California Fine Wire). Both the neural and EKG signals were preamplified, digitized, and recorded using either the Blackrock Microsystems data acquisition system or the Plexon Multichannel Acquisition Processor system. The behaviors of mice were recorded simultaneously. For the Blackrock Microsystems system, local field potentials (LFPs) and EKGs were digitized at 2 kHz and filtered at 1–500 Hz, whereas spikes were digitized at 30 kHz and filtered at 600–6000 Hz. For the Plexon system, LFPs and EKGs were digitized at 1 kHz and filtered at 0.7–300 Hz, whereas spikes were digitized at 40 kHz and filtered at 400–7000 Hz. A total of one to three sites in the BLA were recorded from each mouse.

Experimental design

High-place exposure. The following high-place exposures were used: (1) open high-place exposure (10 × 10 cm, 10 or 20 cm height; Figs. 2A, 3C), in which a mouse was gently picked up from the home cage and placed on the high place for ~3 min (in a few cases ~20 min; see Fig. 5D), then the mouse was picked up again and placed back in the home cage; and (2) open/enclosed high-place exposure (Fig. 4A,B). The test chamber consisted of a waiting room (15 × 20 cm) and a 20-cm-high platform (10 × 10 cm) with or without transparent Plexiglas walls. First, mice stayed in the waiting room for 5–10 min. Then, on opening of the sliding door (by the experimenter), mice could walk freely to the open or

Figure 2. BLA neurons represent fear of heights. **A**, Left, Illustration of a high place (10 × 10 cm; 20 cm height). Right, Activity of one representative BLA neuron in response to the high-place exposure. The red horizontal bar indicates the exposure duration (202 s). **B**, Left, Instant HR recorded simultaneously with the neuron as shown in **A**. Top bars, Red, freezing; blue, not freezing. Right, Poincaré plot of R–R intervals before (gray) and during the high-place exposure (red). R–R interval, the time elapsed between two successive R waves of the EKG signal (see Materials and Methods). **C**, Left, middle, PCA classifies BLA neurons into three categories: high-place fear neurons (light blue; $n = 30$), rebound neurons (orange; $n = 32$), and others (gray; $n = 474$) based on z -scored activity (right panel). PC1, PC2, and PC3 represent the first three principal components color coded from low scores (dark blue) to high scores (light yellow). Neurons in left, middle, and right panels are arranged in the same order. The z score transform was based on the mean and SD calculated between -2 and -0.5 min. **D**, Averaged responses of the high-place fear neurons (light blue; $n = 30$; mean $\pm$ SEM), rebound neurons (orange; $n = 32$), and others (gray; $n = 474$). **E**, Mice showed a significant increase in HR and decrease in HRV on high-place exposure ($n = 5$). HRV was measured by CV of the instant HR. **F**, Mice exhibited a significant increase in freezing during the high-place exposure ($n = 13$). In box plots, the central red lines indicate the median value, whereas the box edges and whiskers mark the interquartile ranges and limits, respectively. $**p < 0.01$; $***p < 0.001$; paired t test.

enclosed high platform. Once the mouse was on the high platform, the sliding door would be closed for ~ 3 min. On the open high platforms, mice typically showed increased heart rate, freezing, and stretch-attend posture (a forward elongation of head and shoulders followed by retraction to the original position).

Modality-specific fear test. The following modality-specific fear tests were conducted: (1) the loud sound stimulus consisted of 10 pips (2 kHz; 100 ms; 90 dB) delivered at 1 Hz (note: loud white noise is also highly effective in evoking BLA neural response and behavioral changes); (2) the looming stimulus was an overhead fast-approaching round disk (12 cm in diameter; delivered by the experimenter); (3) the cat odor stimulus was delivered by placing a live cat next to the mouse chamber (no physical or visual contact); and (4) the high place was a 20-cm-high platform. Note that all of the above tests (except for the cat odor test) typically evoked consistent freezing across animals. For the cat odor test, behavioral responses largely varied across animals, probably because of the use of different cats: one wild-like cat (preying on mice) is highly effective in inducing freezing in mice (in this case, simply using the cat bedding induced long-lasting freezing), whereas other cats (i.e., pets) barely induced any freezing or noticeable behavioral changes in mice. Therefore, we only included data from two mice that showed clear freezing to cat odor for analyses (Fig. 5E,F).

Open field and enriched box exposure. A round chamber (40 cm in diameter, 35 cm in height) was used as the open field arena; a rectangle box (34 × 44 × 38 cm or 40 × 48 × 48 cm, divided into six or eight small rooms and enriched with objects/toys) was used as the enriched box. Mice were manually transferred from home cages to the open field arena or enriched box for a 30 min free exploration and then placed back to home cages.

Fear conditioning. The fear-conditioning chamber was a square chamber (25 × 25 × 32 cm) with a 36-bar inescapable shock grid floor (Med Associates). The behaviors of mice were recorded by using the Blackrock Microsystems NeuroMotive System or the Plexon CinePlex video-tracking system (30 frames/s). On the training day (day 1), mice were allowed to explore the conditioning chamber for ~ 3 min. Then a conditioned stimulus (CS), consisting of 10 100 ms pips (75 dB, 2 kHz) or 10 100 ms flashing lights repeated at 1 Hz, was delivered, followed by an unconditioned stimulus (US; a continuous 0.5 s footshock at 0.75 mA) at the termination of the CS. This CS–US pairing was repeated five times, with a 2 min interval between trials. On day 2, mice were placed back in the conditioning chamber for a 5 min contextual recall test, and the total time of freezing for each mouse was measured. Approximately 10 min later, we conducted the cued recall test in a novel chamber, as follows: mice were allowed to freely explore for 3 min before

Figure 3. BLA high-place fear neurons exhibited little adaptation and were insensitive to different heights. **A**, Activity of the same neuron as shown in Figure 2A on high-place exposure on days 1, 2, and 3. The durations of high-place exposure were indicated by red solid lines (202, 186, and 173 s, respectively). **B**, BLA high-place fear neurons showed significant activation above home cage baseline on high-place exposure ($n = 13$ from 8 mice). *** $p < 0.001$, one-way repeated-measures ANOVA followed by Bonferroni *post hoc*. No difference was observed across the 3 d ($p = 1.000$). **C**, Activity of one representative BLA high-place fear neuron in response to different heights from 5 to 20 cm. Left, The 5-cm-high place: this mouse voluntarily climbed up and down (4 and 11 s for the first and second trials, respectively). Middle, right, The 10- and 20-cm-high places with durations of 193 and 216 s, respectively. **D**, BLA high-place fear neurons showed robust activation to both 10 and 20 cm heights ($n = 13$ from 9 mice). *** $p < 0.001$, one-way repeated-measures ANOVA followed by Bonferroni *post hoc* test. There was no significant difference between the 10- and 20-cm-high place exposures ($p = 1.000$). One outlier is shown (blue cross). For the 5 cm height, the data were not sufficient for statistical analysis because the mice would climb down easily, often within a few seconds.

the onset of the 10 s CS (repeated seven times with 1.5–2.5 min randomized intertrial intervals; no US was delivered). The CS-induced freezing was measured during the first 30 s after onset of the CS.

The above behavioral tasks were performed across 5 d, and the number of animals used in each task was specified in the figure legends. We started with the high-place exposure test (often including the different heights of exposure and open/closed high-place exposure; day 1), followed by the open field and enriched box test (day 2), the modality-specific fear test (day 3), and last, the fear-conditioning test (days 4 and 5). The total number of recorded BLA high-place fear neurons vary across the above tests for the following two main reasons: (1) because of the challenge in stably recording any single neuron across days, some high-place fear neurons disappeared after 1 d or a few days of recording, but, on the other hand, a new high-place fear neuron sometimes emerged on the second day or later of recording; and (2) for some animals, we skipped one or a few behavioral tests for which we had collected sufficient data for statistical analyses (for example, the open field and enriched box test on day 2), which allowed us to prioritize the collection of much needed data on later-day tests (e.g., the fear-conditioning test on days 4 and 5).

Data analysis

The recorded spike activities were sorted by using the MClust 3.5 program (<http://redishlab.neuroscience.umn.edu/MClust/MClust.html>) or the Plexon Offline Sorter, and sorted spikes were further analyzed in NeuroExplorer (NexTechnologies) and MATLAB (MathWorks). The z score values were calculated by subtracting the mean baseline firing rate established over a defined period preceding the stimulus onset from individual raw values (Fig. 2, legend; and see Fig. 7, legend). Then, the difference was divided by the SD calculated from the same period. For measuring the population response significance, neural activity was

calculated by comparing the firing rates before and during the stimulus using a z score transform (bin size, 1 s).

The principal component analysis (PCA) was conducted in MATLAB, similar to that described previously (Opalka et al., 2020). We first extracted the three major principal components (PC1, PC2, and PC3), and then used a hierarchical clustering algorithm (Linkage) to find the similarities (Euclidean distance) among all pairs of z-scored firings in principal components space, iteratively grouping the z-scored firings into larger and larger clusters based on their similarity. Last, we set a distance criterion to extract three clusters from the hierarchical tree (Fig. 2C).

HR and HR variability (HRV) analyses were similar to that described previously (Liu et al., 2013, 2014). Briefly, the typical peaks in heart beats were identified with the timestamps of R-wave peaks. The discrete timestamps of R-wave maxima were obtained by a peak detection algorithm, and the R–R intervals were converted into instant HR (beats per minute). The variability of R–R intervals was graphically described as a Poincaré plot. Each heartbeat interval, the R–R interval_n, was plotted on the x-axis against the subsequent heartbeat interval, the R–R interval_{n+1}, on the y-axis. The coefficient of variation (CV) of instant HR was based on the following formula: $CV = (SD/\bar{x}) \times 100\%$, where $\bar{x}$ (mean) and SD were the $\bar{x}$ and SD of instant HR.

Freezing was defined by the total absence of movement except breathing. We manually scored the freezing. First, we visually inspected the recorded video and identified potential freezing episodes. Second, we screened each potential freezing episode frame by frame (30 frames/s) to determine the length of freezing. A mouse was considered to be freezing if a freezing episode lasted ≥ 2 s. The length of each freezing episode was added, and the percentage of freezing was then calculated (note: the freezing of three mice during the high-place exposure was not included in our analysis because of the malfunction of the video recording).

Figure 4. BLA high-place fear neurons differentially respond to open and enclosed high places. **A**, **B**, Left panels, Illustration of an open (**A**) and an enclosed (**B**) transparent high place. Middle and right panels, Activity of two simultaneously recorded BLA neurons in response to the open (**A**) and enclosed (**B**) high-place exposure. The red horizontal bars indicate the durations of the open and enclosed high-place exposures (200 and 195 s, respectively). **C**, Another representative high-place fear neuron exhibited robust firing increases when the enclosed high place was converted into an open high place. **D**, Mean activity of high-place fear neurons ($n = 10$ from 6 mice) under baseline, enclosed, and open high-place exposure conditions. *** $p < 0.001$, one-way repeated-measures ANOVA followed by Bonferroni *post hoc*.

Statistical analysis

For the comparisons of multiple means, one-way repeated-measures ANOVA and *post hoc* Bonferroni tests were conducted to assess the difference of means. Differences between two means were assessed with paired *t* tests. All statistical comparisons were performed using OriginPro Software (OriginLab). Data were summarized using box plots showing median values and interquartile ranges. The default value of whiskers in the boxplot correspond to $\pm 2.7\sigma$ and 99.3% coverage of the normally distributed data as per the data analysis tool in MATLAB. Differences were considered significant if *p* values were < 0.05 .

Histology

At the end of the recording session, mice were anesthetized, and the final positions of the tetrode bundles were marked by applying an electrical current (10 μ A, 15 s) through two tetrodes. Then, mice were intracardially perfused with PBS followed by 4% paraformaldehyde (PFA) or 10% formalin. Brains were removed and postfixed in 4% PFA or 10%

formalin, allowing ≥ 24 h before slicing on a vibratome (50 μ m coronal sections; Leica). The actual electrode positions were confirmed by histologic staining using the antifade mounting medium with DAPI (Vector Laboratories).

Results

Identification of BLA high-place fear neurons

We unilaterally implanted a 32- or 64-channel tetrode array into the BLA to monitor neuronal activities in freely behaving mice (Fig. 1A; see Materials and Methods). Handling procedures, such as picking up a mouse by its tail and restraining it in the experimenter's hands, are widely used in standardized animal protocols (Deacon, 2006; Leach and Main, 2008). However, this routine practice induces significant fear/stress responses, such as increases in HR, blood pressure, and stress hormone levels (Balcombe et al., 2004); thus, to minimize the

Figure 5. Modality-specific BLA fear neurons. **A–D**, Activity of four simultaneously recorded BLA neurons in response to loud sound (**A**), looming object (**B**), cat odor (**C**), and high-place exposure (**D**). Note that each neuron responded preferentially to only one of the four fear-related stimuli in a long-lasting manner. The loud sound consisted of 10 pips (2 kHz; 100 ms; 90 dB) delivered at 1 Hz; the looming stimulus was an overhead fast-approaching object; the cat odor was delivered by placing a live cat next to the mouse chamber (no physical or visual contact); and the high place was a 20-cm-high platform. **E**, Mean activity of high-place fear neurons under baseline, high-place, loud sound, looming object, and cat odor exposure conditions ($n = 14$ from eight mice for all except the cat odor, in which only 3 of the 14 neurons were tested; see Materials and Methods). *** $p < 0.001$, one-way ANOVA followed by Bonferroni *post hoc*. **F**, BLA neurons that responded to loud sound ($n = 7$ from four mice), looming object ($n = 10$ from eight mice), or cat odor ($n = 3$ from two mice) showed no response to height threats. Mean firing rates were calculated during the first 20 s, 10 s, and 20 min immediately after stimulus onset, respectively. *** $p < 0.001$, paired *t* test.

stress level of the animals during our chronic *in vivo* recording, we used an alternative handling method known as cup handling (Fig. 1*B*, left). In this method, a mouse is scooped up and allowed to walk freely over the experimenter's open hands without direct physical restraint (Hurst and West, 2010). We recently reported that this cup-handling procedure also led to a robust increase in HR and a decrease in HRV, even after repeated handlings over many days (Liu et al., 2013). Correspondingly, we noticed that a subset of BLA neurons exhibited robust firing increases on cup handling, accompanied by an increased HR and decreased HRV (Fig. 1*B*, right). These BLA neurons showed little adaptation to repeated cup handlings (Fig. 1*B*), in contrast to a typical rapid

adaptation of BLA responses to other external stimuli (Bordi and LeDoux, 1992; Breiter et al., 1996; Wei et al., 2015). This lack of adaptation suggests that the cup handling induced BLA activation, and physiological changes could be elicited by heights rather than the procedure itself.

To confirm that the BLA neurons indeed respond to height threats, we designed a 20-cm-high Plexiglas platform to minimize human interference (Fig. 2*A*, left). We placed the mouse on the high platform for ~3 min and confirmed that the cup handling-responsive BLA neuron exhibited a sustained activation during the entire exposure period on the high platform (Fig. 2*A*, right; the same neuron as shown in Fig. 1*B*). This BLA neuronal

Figure 6. BLA high-place fear and anxiety neurons. **A**, A representative BLA high-place fear neuron exhibited robust increased firing on exposure to the high place (left), but not to the open field (middle) or enriched box (right). Note that there were brief activations around Time 0 and 30 because of cup handling (middle and right panels). The intervals between the sessions were 1–2 h. **B**, Mean activity of individual high-place fear neurons (red circles) when the mice were exposed to the high place, open field, or enriched box ($n = 21$ from nine mice). **C**, A representative BLA anxiety neuron exhibiting a slow onset (~ 10 min) of increased firing in the open field (middle), but not on the high place (left) or in the enriched box (right). **D**, Mean activity of the anxiety-related neurons when the mice were exposed to the high place, open field, or enriched box ($n = 13$ from six mice). **E**, A representative BLA rebound neuron exhibiting general excitation following removal from the high place (left), open field (middle), or enriched box (right). **F**, Mean activity of the rebound neurons when the mice were placed back into the home cage from the high place, open field, or enriched box ($n = 20$ from eight mice). The box plots were similar to that shown in Figure 1, except that the blue crosses indicate outliers. *** $p < 0.001$, one-way repeated-measures ANOVA followed by Bonferroni *post hoc* test.

activity was accompanied by an increased HR, a decreased HRV, and increased freezing (Fig. 2B), indicating a representation of fear of heights (Miyata et al., 2007). Therefore, we mainly used this high-place (20 cm) exposure procedure to determine the responses of BLA neurons to height threats.

In total, we recorded 536 BLA neurons from 16 mice on exposure to height threats. To identify major population activity patterns, we classified these BLA neurons based on z-scored activity using an unsupervised method: the PCA. Three major categories were classified (Fig. 2C,D). Approximately 5.6% of the BLA neurons (30 of 536 neurons; light blue) that exhibited a significant increase in firing on the high platform were named “high-place fear neurons” (Fig. 2C,D; average z score, >3.29 ; $p < 0.001$). A second subset of BLA neurons (6.0%, 32 of 536 neurons; orange) that showed an excitation following removal from the high platform were named “rebound neurons” (Fig. 2C,D; average z score, >3.29 ; $p < 0.001$). And the remaining BLA neurons (474 of 536; gray) were termed “other neurons” (Fig. 2C,D). We noticed that some of these other neurons exhibited a

decrease in firing by visual inspection; however, they cannot be classified into a major category because of the relatively small number and low baseline firing rates (which would prevent meaningful comparison given the one-trial nature of the high-place exposure test). This is consistent with previous studies reporting the challenge in measuring inhibitory responses of BLA neurons (Quirk et al., 1995; Chen and Sara, 2007; Grimsley et al., 2013; Bارسy et al., 2020).

Accordingly, this fear state induced by the height threat was confirmed in a subset of animals, evidenced by a significant increase of HR (Fig. 2E; paired t test: $t_{(4)} = -7.053$, $p = 0.002$), a reduction of HRV (Fig. 2E; paired t test: $t_{(4)} = 6.635$, $p = 0.003$), and an increase in the duration of freezing during the high-place exposure (Fig. 2F; paired t test: $t_{(12)} = -9.255$, $p = 8.208 \times 10^{-7}$). For the following experiments and analyses, initially we focused on discussing BLA high-place fear neurons whose responses are highly selective to height threats, and later we included BLA rebound neurons whose responses are generalized to various environmental changes beyond height threat relief (Fig. 6).

To assess whether BLA high-place fear neurons habituate to height threats, we tested some of the stably recorded neurons for 3 consecutive days. As expected, the high-place fear neurons consistently exhibited robust increases in firing on high-place exposure from day 1 to 3 (Fig. 3A). Our quantitative analyses showed that there was little adaptation in neural firing during this repeated test across days (Fig. 3B; one-way repeated-measures ANOVA: $F_{(2,230,26.76)} = 47.993$, $p = 6.223 \times 10^{-10}$; *post hoc* Bonferroni test: high-place exposure day 1 vs day 2 vs day 3, $p = 1.000$, $p = 1.000$, $p = 1.000$, respectively). Additionally, we tested some high-place fear neurons with different heights of exposure and revealed that even a low height (5 cm) can trigger a robust activation (Fig. 3C, left). At this low height, mice climbed off the 5 cm platform easily; therefore, we limited our analyses to 10- and 20-cm-high platform exposures in which mice typically could not climb off voluntarily. Our results showed that BLA high-place fear neurons exhibited similar activations on the 10- and 20 cm high-place platform, indicating a saturated response once the platform reached a certain height (Fig. 3C,D; one-way repeated-measures ANOVA: $F_{(1,731,20.77)} = 59.599$, $p = 5.924 \times 10^{-9}$; *post hoc* Bonferroni test: 10- vs 20-cm-high place exposure, $p = 1.000$). This corroborates a recent virtual reality study in humans that reported saturated behavioral responses above a specific height threshold (Wuehr et al., 2019).

To determine whether the activation of high-place fear neurons may rely on visual or other sensory inputs, we designed another testing chamber, which consisted of a waiting room attached to a high-place platform (with or without transparent Plexiglas walls; Fig. 4A,B, left panels). First, mice stayed in the waiting room for 5–10 min. Then, after opening the sliding door, the mice could walk freely over to the open high place (Fig. 4A, left) or the transparent enclosed high place (Fig. 4B, left). Once the mice walked onto the high platform, the door was closed, forcing the mice to remain on the open/enclosed high place for ~3 min before being allowed back to the waiting room. Our results revealed that the high-place fear neurons reduced firing (Fig. 4B, middle) or ceased to fire (Fig. 4B, right) on the enclosed high place, compared with the robust responses on the open high place (Fig. 4A, middle, right). To further confirm this response characteristic, we tested some high-place fear neurons in one experimental session (waiting room → enclosed high place → open high place). Consistently, the high-place fear neurons exhibited significant firing increases when the enclosed high place was converted into an open high place (Fig. 4C). Overall, the high-place fear neurons increased firing preferentially on the open high place compared with the enclosed high place (Fig. 4D; one-way repeated-measures ANOVA: $F_{(1,244,11.20)} = 64.265$, $p = 2.939 \times 10^{-6}$; *post hoc* Bonferroni test: $p = 0.283$, $p = 1.115 \times 10^{-8}$, $p = 1.798 \times 10^{-7}$, respectively), suggesting that nonvisual sensory inputs also drive the activation of BLA high-place fear neurons. In support of this, our preliminary observation revealed that the high-place neurons were activated when mice approached the edge of a high place in a completely dark environment (infrared recording). Nonetheless, a subset of BLA neurons clearly increased firing on exposure to the transparent enclosed high place, which was attributed mainly to a visual depth perception of the height (Fig. 4B, middle, D). This suggests that the BLA high-place neurons at least partially receive visual inputs, and that other sensory information such as vestibular inputs may also contribute to their activation on exposure to a high place.

BLA high-place fear neurons do not process various sensory threats

Recent studies have reported that the amygdala plays a central role in processing innate fear of predator odors and looming

visual threats (Wei et al., 2015; Jhang et al., 2018; Salay et al., 2018). Thus, we tested whether the BLA neurons respond on exposure to cat odor (olfactory), looming object (visual), as well as loud sound (auditory). As previously reported, cat odor may or may not induce consistent fear/anxiety responses (Raud et al., 2007; Hacquemand et al., 2013; Arakawa, 2019). Similarly, we also noticed a large behavioral variability among individual mice in response to cat odor; thus, we only included mice that expressed clear freezing behavior for analysis (see Materials and Methods). Our results revealed that a subpopulation of BLA neurons preferentially responded to one of the stimuli, including loud sound (Fig. 5A), looming object (Fig. 5B), or cat odor (Fig. 5C), but not height threats (Fig. 5F: left, $t_{(6)} = 6.235$, $p = 7.876 \times 10^{-4}$; middle, $t_{(9)} = 7.575$, $p = 3.413 \times 10^{-5}$; right, $t_{(2)} = 3.221$, $p = 0.084$; paired *t* test). Similarly, the BLA high-place fear neurons did not respond to any of the above sensory stimuli except for the height threats (Fig. 5D,E; one-way ANOVA: $F_{(4,54)} = 63.225$, $p = 0$; *post hoc* Bonferroni test: baseline vs high place, $p = 1.268 \times 10^{-16}$; high place vs loud sound, $p = 2.702 \times 10^{-17}$; high place vs looming object, $p = 3.500 \times 10^{-17}$; high place vs cat odor, $p = 1.330 \times 10^{-9}$). Together, these results indicate a highly selective response of BLA neurons to distinct fear stimuli.

BLA high-place fear neurons do not process anxiety

While often occurring together, fear and anxiety differ in terms of the following key characteristics (Davis et al., 2010): fear is fast onset and usually triggered by a specific threat, while anxiety is characteristically slow onset and long lasting when no immediate external threat is present. Nonetheless, the brain areas that process fear and anxiety greatly overlap (Tovote et al., 2015), and, in particular, the BLA is a key brain region implicated in both fear and anxiety processes (Blanchard and Blanchard, 1972; LeDoux, 2000; Pitkänen et al., 2000; Tye et al., 2011; Wang et al., 2011). To determine whether the BLA high-place fear neurons were also involved in processing anxiety, we exposed mice to an open field, previously shown to be mildly anxiogenic (Walsh and Cummins, 1976; Prut and Belzung, 2003), an enriched box, consisting of multiple small rooms with toys inside (anxiolytic), and the high platform (Fig. 6A, top row). Our results revealed that most high-place fear neurons exhibited no activations during either open field or enriched box exposure (Fig. 6A,B; one-way repeated-measures ANOVA: $F_{(1,780,35.60)} = 107.936$, $p = 3.096 \times 10^{-15}$; *post hoc* Bonferroni test: $p = 3.473 \times 10^{-14}$, $p = 5.987 \times 10^{-16}$, $p = 0.356$, respectively). On the other hand, another subset of BLA neurons gradually increased their firing (with a slow onset) in the anxiogenic open field, but not in the enriched box or on the high place (Fig. 6C,D; one-way repeated-measures ANOVA: $F_{(1,114,13.37)} = 91.498$, $p = 1.538 \times 10^{-7}$; *post hoc* Bonferroni test: $p = 1.371 \times 10^{-10}$, $p = 1.000$, $p = 3.058 \times 10^{-11}$, respectively), consistent with our previous study that reported anxiety-related neurons in the BLA (Wang et al., 2011). Meanwhile, our results showed that the BLA rebound neurons (Fig. 2C, classification) exhibited a universal excitation pattern following removal from high place, open field, or enriched box (Fig. 6E,F; one-way repeated-measures ANOVA: $F_{(2,568, 48.79)} = 41.264$, $p = 1.479 \times 10^{-12}$; *post hoc* Bonferroni test: rebound after high-place exposure vs after open field exposure vs after enriched box exposure, $p = 0.587$, $p = 1.000$, $p = 1.000$, respectively). This lack of response selectivity suggests that the activation may be triggered by familiarity/safety of the home cage environment or by relief from environmental uncertainty. Overall, the differential response patterns of high-place fear neurons and anxiety neurons suggest that fear of heights and the emotional state of anxiety are, to

Figure 7. BLA high-place fear neurons process contextual fear but not cued fear. **A**, Experimental procedure. Mice received five pairings of tone/light footshock on day 1. On day 2, mice were tested with contextual recall, tone/light recall, and high-place exposure. **B**, Top, A representative high-place fear neuron exhibited a robust increase in firing during the contextual recall (red horizontal bar, 324 s). Bottom, Mean activity of individual high-place fear neurons ($n = 8$ from six mice; red circles) during contextual habituation (before footshock) and contextual recall. $***p < 0.001$, paired t test. **C**, Top, Responses of the same high-place fear neuron (as shown in **B**) to the conditioned tone. Bottom, Averaged activity (mean $\pm$ SEM; z scored) of high-place fear neurons in response to the conditioned tone ($n = 8$). The z score transform was based on the mean and SD calculated between -10 and 0 s. **D**, Top, Responses of the same high-place fear neuron (as shown in **B**) to high-place exposure (red horizontal bar; 314 s). Bottom, Mean activity (mean $\pm$ SEM; z scored) of the same BLA neurons in response to the high-place and conditioning chamber exposures ($n = 8$). The z score transform was based on the mean and SD calculated between -5 and -0.5 min. **E**, The mice exhibited high levels of freezing in both the contextual and cued recall tests ($n = 6$), $***p < 0.001$, one-way repeated-measures ANOVA followed by Bonferroni *post hoc* test. **F**, Correlation analysis of BLA high-place neurons ($n = 8$) between high-place exposure and contextual recall ($n = 8$; $r = 0.72$, $*p < 0.05$, Pearson's correlation coefficient).

a great extent, independently processed by distinct BLA neural populations.

BLA high-place fear neurons process contextual fear

The amygdala also plays an essential role in processing conditioned fear (LeDoux, 2000). To determine whether the high-place fear neurons were involved in processing associative fear formation, we used a classical fear-conditioning procedure (Fig. 7A). Mice, in which at least one high-place fear neuron was recorded, were habituated to a conditioning chamber for 3 min, followed by multiple pairings of tone/light footshock (four mice received the tone-footshock pairings, whereas two other mice received the light-footshock pairings). On the next day, mice underwent contextual and cued (tone/light) recall tests, in addition to a high-place exposure test. Our results revealed that the BLA high-place fear neurons exhibited a robust increase in firing during the contextual recall compared with the 3 min habituation before the footshock (Fig. 7B; paired t test: $t_{(7)} = -6.072$, $p = 5.049 \times 10^{-4}$). This suggests that the high-place fear neurons also process learned contextual fear information. In contrast, the high-place fear neurons showed no firing change in response to the novel cued recall chamber or to the conditioned tone/light (Fig. 7C), indicating a

specificity in cued versus contextual responses of BLA high-place fear neurons. Notably, these dissociated responses to the context and cue did not appear to result from a failure of the conditioning, as all the mice showed high levels of freezing during both the cued and contextual fear tests (Fig. 7E; one-way repeated-measures ANOVA: $F_{(1,369, 6.84)} = 101.815$, $p = 1.418 \times 10^{-5}$; *post hoc* Bonferroni test: $p = 2.876 \times 10^{-7}$, $p = 2.436 \times 10^{-6}$, $p = 0.062$, respectively). In general, BLA high-place fear neurons exhibited comparably high activity on exposure to the conditioning chamber, albeit a gradual decrease in firing across the 5 min (Fig. 7D). Further analyses revealed a positive correlation of the BLA activity between high-place and contextual fear exposures, indicating a convergence in representation of height and contextual threats by the high-place fear neurons (Fig. 7F; Pearson's correlation coefficient: $r = 0.72$, $p = 0.045$). Overall, these results demonstrate that BLA high-place fear neurons also represented learned fear of context (or fear memory) in addition to the fear of heights.

Discussion

Emerging evidence suggests that the amygdala plays a central role in processing innate fear, including fear of predator odors

and looming visual threats (Wei et al., 2015; Jhang et al., 2018; Salay et al., 2018). The neuronal representation of fear of heights, however, has not been explored. In this study, we discovered a discrete set of BLA neurons that responded robustly to height threats. These BLA neurons were activated by the passive placement on and voluntary exploration of a high place, accompanied by increased freezing, increased heart rate, and decreased heart rate variability. This provides the first evidence that the BLA likely engages in processing the innate fear of heights. Additionally, we identified another group of BLA neurons, termed rebound neurons, that can be activated by returning to a familiar/safe home cage environment. This adds to the growing evidence of the role of the amygdala in signaling familiarity and safety (Farovik et al., 2011; Christianson et al., 2012; Genud-Gabai et al., 2013; Sangha et al., 2013; Likhtik et al., 2014).

Among amygdala nuclei, the BLA has been identified as the major site for receiving cortical and thalamic sensory inputs (McDonald, 1998; Aggleton, 2000). Consistent with this anatomic connectivity, the BLA neurons have been shown to respond to a variety of visual, auditory, olfactory, gustatory, and somatosensory stimuli (Bordi et al., 1993; Romanski et al., 1993; Schoenbaum et al., 1999; Liu et al., 2018; Morrow et al., 2019). In turn, this BLA activity leads to emotional and physiological reactions such as freezing and sympathetic arousal (Laine et al., 2009; Shabel and Janak, 2009; Bonnet et al., 2015). Our results largely expanded these earlier findings that typically reported brief activations of BLA neurons (milliseconds to a few seconds) by external sensory stimuli. Specifically, we found that the BLA neurons can also exhibit long-lasting activations (seconds to minutes) that preferentially responded to acoustic startle, looming visual threat, predator odor, or height threat (Fig. 5). This difference could be explained by a predominance of our recording from the basal amygdala, whereas the above-mentioned studies largely targeted the lateral amygdala. Together, our results provide direct evidence that BLA neurons represent various types of innate fear in a modality-specific and likely pathway-specific manner.

Recently, the following two thalamic pathways of visual looming threat inputs to the BLA have been identified: one circuit from the superior colliculus via the lateral posterior nucleus to the BLA (Wei et al., 2015); and the other circuit from the xiphoid nucleus to the BLA (Salay et al., 2018). These findings provide possible neural pathways for activation of the BLA neurons triggered by the looming threat (Fig. 5B,F), but likely not the high-place fear neurons because of their insensitivity to the looming threat (Fig. 5D,E). Given that visual depth perception is important for the generation of fear of heights (Gibson and Walk, 1960; Fox, 1965; Teachman et al., 2008), other unidentified visual pathways may contribute to the activation of BLA high-place fear neurons, such as those from secondary visual cortices to the BLA. In support of this notion, a subset of high-place fear neurons fired on exposure to a transparent enclosed high place where only visual perception of height is present, and without immediate danger (Fig. 4B,D). Nonetheless, the reduced firing in the enclosed high place suggests that BLA high-place fear neurons also receive other important sensory information in addition to visual inputs, such as a vestibular or visual-vestibular interaction input, to generate the fear of heights (Coelho and Balaban, 2015). Vestibular information processing in rodents seems to be widespread across many brain regions, including sensory cortices (somatosensory and visual), frontal cortices (prefrontal and cingulate), motor cortices, head direction-related regions (hippocampus and retrosplenial cortex), and some

thalamic nuclei (Rancz et al., 2015). Most of these brain regions communicate with the BLA, either directly or indirectly, but it is unclear which region (or regions) may contribute to the activation of BLA high-place fear neurons. Congruent with this notion, a physical therapy study showed that vestibular exercise can improve acrophobia symptoms (Whitney et al., 2005). Another possibility for the reduced responses in the enclosed high place is a regulation by cortical inputs, such as from the anterior cingulate cortex, which processes vestibular information and can inhibit innate fear responses (Jhang et al., 2018).

A large body of research suggests that the BLA is a hub of multiple brain circuits in processing both fear and anxiety (Blanchard and Blanchard, 1972; LeDoux, 2000; Pitkänen et al., 2000; Tye et al., 2011; Calhoun and Tye, 2015; Tovote et al., 2015). Given that fear and anxiety are two distinct emotional states with different behavioral responses (Calhoun and Tye, 2015), it is conceivable that distinct BLA neurons differentially process fear and anxiety. In support of this notion, our results showed that two subpopulations of BLA neurons encoded the fear of heights and the emotional state of anxiety, respectively (Wang et al., 2011). Immediately downstream of the BLA, the ventral hippocampus may receive inputs from the BLA anxiety neurons and promote anxiety (Felix-Ortiz et al., 2013), whereas the central amygdala may receive inputs from the BLA high-place neurons and promote fear (Tovote et al., 2015). This dissociated processing of anxiety and fear of heights is consistent with previous findings that reported no correlation between trait anxiety and fear of heights (Coelho and Wallis, 2010). Nonetheless, a few BLA neurons exhibited activation during both high-place and open field exposure (Fig. 6B, three outliers) indicating a dual representation of fear and anxiety by a small group of BLA neurons. This dual representation could be enhanced, which may explain why fear and anxiety become linked under extreme conditions such as pathologic fear. In fact, a new subcategory of anxiety disorder, termed visual-vestibular fear has recently been proposed (Coelho and Balaban, 2015).

It is believed that the amygdala is essential in processing both cued and contextual fear (Phillips and LeDoux, 1992). A number of studies has shown that the cued fear plasticity occurs predominantly in the BLA (LeDoux, 2000; Paton et al., 2006; Herry et al., 2008; Beyeler et al., 2016) through cortical and thalamic sensory inputs (Tovote et al., 2015). On the other hand, contextual fear likely recruits the medial entorhinal cortex–BLA pathway (Kitamura et al., 2017) since the BLA has been implicated in both the acquisition and retrieval of contextual fear (Sparta et al., 2014; Xu et al., 2016). What remains unclear is whether the same population or two populations of amygdala neurons are involved in processing cued versus contextual fear. Our results revealed that, after fear-conditioning training, the BLA high-place fear neurons exhibited a selective response to the context but not to the cue (auditory or visual), suggesting that the fearful context and cue are independently processed by the BLA neurons. Although the high-place fear neurons should at least partially receive visual inputs (Fig. 4B,D), their lack of response plasticity to the conditioned visual cue suggests that the visual perception of light and visual perception of height/depth are mediated by different neural pathways. This response selectivity is also consistent with our finding that the BLA high-place fear neurons did not respond to fear-related sensory stimuli, including loud sound, looming threat, and predator odor (Fig. 5E).

In summary, we discovered a discrete set of BLA neurons that respond to both high-place and fear context exposure, indicating a convergence in processing of dangerous/risky contextual

information. These BLA high-place fear neurons likely receive strong visual–vestibular information from sensory inputs but relatively weak spatial/contextual information from entorhinal–hippocampal inputs: the former exhibit little habituation or plasticity change across multiple trials, whereas the latter can be readily enhanced by a fear-conditioning procedure likely through a long-term potentiation mechanism. Our results provide insights into how fear of heights is processed in the brain, which may have clinical implications for the treatment of excessive fear and anxiety disorders, such as acrophobia.

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