Looming stimuli reliably drive innate, but not learned, defensive responses in rats.

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Abstract

The ability to respond innately to stimuli such as food, water, and threat is crucial for survival as it guarantees the ability to feed, drink, and avoid danger without prior experience. These natural, unconditioned, stimuli can also drive associative learning, such that cues consistently paired with an unconditioned stimulus come to elicit a response. Threat conditioning, an extensively studied form of associative learning, typically uses painful electric shocks that mimics injury as unconditioned stimuli. Whether injury or pain is required for learning, or the threat of injury suffices, remains elusive. An approaching predator produces looming shadows and sounds. Visual looming stimuli elicit strong innate defensive responses such as escape and freezing. Here we ask whether these stimuli can also drive learned freezing or escape responses to a conditioned stimulus. We found that pairing a neutral tone with a looming stimulus failed to drive learned defensive responses, either freezing or escape, in contrast with the robust learned responses when the loom was replaced by shock. The dissociation

between the capacity to drive an innate defensive response and to drive threat learning reveals new boundaries for learned defensive responses which will impact our understanding of learning processes and defensive strategies both at the mechanistic and ethological levels.

Key words: fear, threat conditioning, learning, looming stimuli, defensive behaviors, freezing, escape

Introduction

Most of our knowledge regarding survival circuits in the brain comes from threat conditioning studies (Gross & Canteras, 2012; LeDoux, 2000; Tovote, Fadok, & Lüthi, 2015). These studies mainly focus on defensive behavioral responses, such as freezing, to learned cues predicting an aversive stimulus. Electric shocks, which mimic painful injury upon contact with a predator, are predominantly used as the unconditioned aversive stimulus. More recently, other noxious stimuli have been shown to effectively drive this form of learning, such as heat (Biederman & Davey, 1997; Hendersen & Graham, 1979; Kung, Su, Fan, Chai, & Shyu, 2003), or activation of pain responsive brain regions (Han, Soleiman, Soden, Zweifel, & Palmiter, 2015). Non-painful threatening stimuli are seldomly used for threat learning, even though animals can rapidly detect danger in the form of a predator through visual, chemical and auditory cues, and avoid contact through rapid innate defensive responses like freezing and escape (Pereira & Moita, 2016). Whether these predator-associated cues are able to drive learning remains unclear. Predator odors have been used to drive threat learning with variable results. In most cases the context in which subjects were exposed to predator odors fails to drive freezing or escape responses, although sometimes it drives avoidance behaviors (Blanchard, Yang, Li, Gervacio, & Blanchard, 2001; Takahashi, 2014; Wallace & Rosen, 2000). One possibility is that a predator's odor, often present in excretions, does not imply the predator's proximity, thus constituting a remote cue of threat. Hence, animals may learn to avoid the location where the odor was scented, but will not learn to exhibit acute defensive responses like escape or freezing, typically triggered by an imminent encounter with a predator.

A looming stimulus, in the form of a rapidly expanding black disk that mimics the shadow of an approaching predator, elicits strong innate freezing and escape responses in all visual animals tested in the lab, including rodents and humans (Ball & Tronick, 1971; Dunn et al., 2016; Oliva, Medan, & Tomsic, 2007; Schiff, Caviness, & Gibson, 1962; Yilmaz & Meister, 2015; Zacarias, Namiki, Card, Vasconcelos, & Moita, 2018). The immediacy of threat associated with a looming stimulus may result in animals learning more effectively to associate cues and locations with its presence and to display acute defensive responses to these cues. Furthermore, different studies have shown projections from the superior colliculus, where information about visual looming stimuli is processed in the brain, to the amygdala (C. Shang et al., 2015; Usunoff, Schmitt, Itzev, Rolfs, & Wree, 2007; Wei et al., 2015), which is thought to play a major role in threat learning (LeDoux, 2000; Tovote et al., 2015).

To test the efficacy of looming stimuli in driving threat learning, we developed a conditioning protocol where a neutral pure tone, the conditioned stimulus, was either paired with foot shock or with a visual looming stimulus that robustly induces freezing and escapes. We previously demonstrated that looming stimuli do not efficiently drive contextual threat learning (Cruz, Heinemans, Márquez, & Moita, 2020), therefore, in this study, we chose a cued conditioning task that typically drives conditioned responses more reliably than contextual conditioning paradigms. We used this conditioning protocol in two separate experiments – one focused on freezing, the other on escape – to assesses whether rats exhibit either of these acute defensive behaviors in response to tones associated with visual looms.

Methods

Subjects

Naïve male Sprague Dawley rats, weighing 225g to 250g, were obtained from Charles Rivers Laboratories (France). Upon arrival, the animals were pair-housed in Plexiglas top filtered ventilated cages (GR900 for rats, Tecniplast S.p.A, Italy) with *ad libitum* access to water and food. They were maintained on a 12h light/dark cycle (lights off at 8 p.m.), a temperature of 20-22°C and 40-70% humidity. After a one-week acclimatization, the experimenter handled all animals on three consecutive days in the week preceding experimental procedures. All animal procedures were performed under the guidelines of the Animal Welfare Body of the Champalimaud Research (Portugal) and in strict accordance with the European Community's Council Directive (86/609/EEC).

Behavioral apparatus

<u>Shock-conditioning:</u> the shock conditioning box (model H10-11RTC, Coulbourn Instruments) was equipped with a metal grid floor to deliver foot shocks (model H10-11RTC-SF, Coulbourn Instruments) and placed inside a sound isolation chamber (Action, automation and controls, Inc) with white walls. The side walls of the conditioning box were made of clear Plexiglas and cleaned with rose scented detergent after every conditioning round. A precision programmable shocker (model H13-16, Coulbourn Instruments) was used to deliver foot shocks. Pure tones (5kHz, 60dB) were produced by a sound generator (RM1, Tucker-Davis Technologies) and delivered through a horn tweeter (model TL16H80HM, VISATON). The rats' behavior was tracked

by a video camera mounted on the ceiling of each sound attenuating chamber. An infrared surveillance video acquisition system was used to record and store all videos on a hard disk and freezing behavior was scored manually offline.

Loom-conditioning: the visual loom conditioning box was made of a black acrylic floor with clear, dark red sides (30cm width x 50cm height x 55cm depth) and was cleaned with a lemon-scented detergent solution. This box was placed in a room with ceiling lights on. Pure tones were delivered through the same system as the shock conditioning (see above). Visual stimuli were projected with an LED projector (ML750e, Optoma Europe ltd, United Kingdom) onto an opaque white Plexiglass screen placed on top of the behavioral box. The behavior was captured with an infrared camera (PointGrey Integrated Imaging Solutions GmbH, Germany) and stored on a hard disk for offline manual scoring. Both the tone-loom delivery and the video capture were controlled by a custom workflow using the Bonsai visual programming language (Lopes et al., 2015).

Recall-test for freezing: the box to test conditioned freezing consisted of a chamber made of clear Plexiglas walls (30cm width x 34cm height x 27cm depth, Gravoplot). The floor contained a removable tray with bedding (the same used in the animals' home cages). The box and tray were cleaned with water and 70% ethanol. The box was placed inside a sound attenuation chamber (80cm width x 52.5cm height x 56.5cm depth) made of MDF lined with high-density sound attenuation foam (MGO Borrachas Tecnicas) and a layer of rubber. Pure tones were delivered through the same equipment as described for conditioning (see above). The behavior of the animals was tracked by infrared video cameras mounted on the walls of the sound attenuating

chambers. A surveillance video acquisition system was used to record and store all videos, and freezing behavior was scored using the FreezeScan software from Clever Sys. In all tests, the rats were considered to be freezing if they did not show any movement except breathing for at least one second.

Escape runway: a large runway (200cm length x 50cm width x 50cm height), with an adjacent shelter (30cm length x 50cm width x 50cm height) was used to look at escape behavior. The shelter consisted of a black acrylic floor, with red-transparent acrylic walls. A black acrylic plate was used as a roof, and could be removed to place rats in the shelter at the beginning of each experiment. The shelter was connected to the large runway through a small gate (10cm x 10cm) that could be closed with a transparent acrylic sliding door. The runway's back and side walls, as well as the floor, were made of (waterproof) black painted wood. The front wall was made out of red-transparent acrylic, allowing for video recording of the rats' behavior inside of the runway, and the ceiling consisted of transparent acrylic with white baking paper on top that functioned as a screen for the looming stimuli. Looming stimuli were projected with an LED projector (ML750e, Optoma Europe ltd, United Kingdom), and pure tones were delivered with a horn tweeter (model TL16H80HM, VISATON). The runway had two infrared lights at each far-end side illuminating the area, while the shelter had one on top. The behavior was captured by infrared cameras (PointGrey Integrated Imaging Solutions GmbH, Germany) and stored for later use. There was one sideways camera capturing the behavior of the rats in the large runway, and one filming the behavior in the shelter from above.

Behavioral procedures

Conditioned freezing experiment

Habituation and tone conditioning: on days 1-3 all rats were exposed to one environment per day: the test box, the loom box, and the shock box, for 20 minutes. Afterwards, the animals were randomly assigned to either the tone-loom or tone-shock conditioning group. On day 4, rats in the tone-shock group were placed in the shock conditioning box, where they received 10 tone-shock pairings after a ten-minute baseline. The pure tones (5kHz, 60dB) lasted 10 seconds, immediately followed by a shock of 0.5mA lasting 0.5s. The interval between tone-shock presentations ranged from 1 to 5 minutes, with an average of 3 minutes. The animals in the tone-loom conditioning group were placed in the loom box, where they received 10 tone-loom pairings after a ten-minute baseline period. The tone had the same properties as that of the tone-shock pairings and was immediately followed by the looming stimulus: a black disk that increased exponentially from 1cm to 30cm in 0.5 seconds. Again, inter-trial intervals varied between 1 and 5 minutes (average of 3 minutes). The behavior of all rats was recorded for offline scoring of freezing.

Conditioned freezing test: the day after conditioning, all animals were placed in the test chamber individually, and after a 5-minute baseline they were exposed to three tones (same as described above) with a 3-minute inter-trial interval. The behavior of the rats was recorded for offline scoring of freezing.

Escape experiment

Habituation: each day after handling (3 minutes for 3 days in the week prior to the start of the experiment), rats received yogurt-flavored pellets (mini yogurt drops, BioServ, United States) in their home cage, to habituate them to the treat. Prior to the beginning of the experiment, rats were habituated to the shelter. Habituation was achieved by placing the rats in the shelter with 3 pellets for two consecutive days, and allowing twenty minutes for rats to explore the closed shelter and eat the pellets. On the first day of the experiement (training day 1) a pellet was placed in the runway, 25cm away from the shelter exit. After putting the rat in the shelter, the door to the runway was opened, and the rat was free to explore the runway and retrieve the pellet. As soon as the first pellet was retrieved and consumed, the sliding door was closed again while the rat was in the shelter, and a second pellet was placed at a 50cm distance from the shelter. The rat was once again allowed to explore and retrieve the pellet, and the sequence was repeated to place a third pellet at a distance of 75cm. The training session ended as soon as the animal had retrieved all three pellets, or if the total time of the session had reached 30 minutes. The second training session, the following day, was identical to the first, with the exception that the pellets were placed at a distance of 50, 75, and 100cm from the exit of the shelter. All training sessions were recorded with the Bonsai visual programming language (Lopes et al., 2015) and saved for offline analysis.

Conditioning: on day 3 of the experiment, the rats were assigned to either a loom-alone, tone-shock, tone-loom, or tone-alone condition. Loom-alone rats, used to

test innate escape responses the looming stimuli, were put in the shock conditioning box for the same length of time as all other animals, but no stimuli were presented. The conditioning protocol for the tone-shock and tone-loom groups was performed as described for the conditioned freezing experiment (see above). In short, after a 10-minute baseline, animals were either exposed to ten tone-shock pairings, or ten tone-loom pairings, with an interval between 1 and 5 minutes (3-minute average). The animals in the tone-alone condition were placed in the shock conditioning box, and received ten pure tone presentations. The interval between the tones was the same as described for the other groups. The behavior of the animals was recorded and saved for offline scoring of freezing. In this experiment, the tones were 1 second rather than 10 seconds long, as a pilot experiment showed that with a 10-second conditioned tone, animals had time to retrieve the pellet and reach the shelter before the end of the tone (when the unconditioned stimulus was expected), whereas the 1-second tone did not allow for this.

Escape test: the day after the conditioning - day 4 - a pellet was placed in the runway at 100cm from the shelter. Afterwards, rats were placed in the shelter and the door to the runway was opened, allowing rats to freely explore the arena, like the pellet retrieval training sessions on days 1 and 2. However, this time rats triggered a stimulus as soon as they reached a 90cm distance from the shelter. For the rats in the loom-alone group, this stimulus was a series of five visual looming stimuli, with an inter-stimulus interval of 1 second. The looming stimuli expanded from 1cm to 30cm in 0.5 seconds. The rats in the three remaining groups received a series of five 1 second pure-tone stimuli (5kHz), with a 1s inter-stimulus interval. The automatic triggering of the

stimuli and the recording of the behavior was done using the Bonsai visual programming language (Lopes et al., 2015).

Criteria for animal exclusion

For the freezing experiment, there were a total of 10 rats in the tone-shock condition, and 18 in the tone-loom condition. In each condition, 2 animals were excluded due to high freezing levels during the baseline, making it impossible to infer the effect of the conditioned tone on their freezing behavior. The behavior of 8 and 16 animals respectively was analyzed. For the escape experiment, 32 animals were used in total. Of those, 7 rats did not manage to retrieve all three pellets on training day 2, and 1 rat managed to open the sliding door by itself during the test, leading to the exclusion of these 8 animals.

Video analysis

The freezing behavior during the cue test of the conditioned freezing experiment was scored automatically using FreezeScan from Clever Sys. This requires optimization and validation, which depends on the camaras used, size of the boxes and illumination, which was previously done for the cue testing chambers (Pereira, Cruz, Lima, & Moita, 2012). Given the variation in all these settings for the conditioning sessions (tone-shock, tone-loom and tone alone) it was difficult to standardize the freezing-settings across conditions. Hence manual scoring was used instead. Videos obtained from the training and test of the escape experiment were used to analyze various behaviors (particularly displacement and pellet retrieval) of the rats. Pellet retrieval, freezing, scanning, and the

time spent in the shelter was scored manually using the open-source program Python Video Annotator (https://pythonvideoannotator.readthedocs.io). The position in the arena of each animal for the duration of training and test was determined using Bonsai visual programming language (Lopes et al., 2015). Information from manual scoring and automated scoring was combined with custom Python (Spyder v3.6) scripts and further analyzed.

Statistical analysis

Statistical analyses were performed using the PRISM 8 software (Graphpad). For the conditioned freezing experiment, differences between groups were investigated using a *Mann-Whitney U* test. Within subject changes in freezing from baseline to stimulation period were investigated with a *Wilcoxon signed-rank* test. In the escape experiment, a *Kruskal-Wallis* analysis with post hoc Dunn's test for multiple comparisons was done to investigate the difference between time spent freezing during conditioning of the three groups. Within-subject changes in time spent freezing were again analyzed with a *Wilcoxon signed-rank* test. Statistical significance was accepted at $p\text{-value}<0.05$ for all tests. Regarding the pellet retrieval in the escape experiment, comparisons between the tone-shock, tone-loom and tone-alone groups were done using a *Kaplan-Meier* test for survival (of the pellet in this case).

Results

Looming stimuli are weak drivers of learned freezing responses

The first experiment was performed to assess the amount of learned freezing triggered by a tone previously associated to either a shock or a visual loom. Rats were put in a conditioning box and exposed to a neutral tone followed by either a foot shock or a visual looming stimulus (fig. 1A). In the conditioning session of this experiment, rats in both groups displayed similarly low freezing during the baseline period (median freezing of shock condition: 1.87%; median freezing of loom condition: 4.35%; $U=251$, $p=0.92$) and showed equivalently high levels of freezing during the tone-shock or tone-loom pairings (fig. 1B, median percentage increase in freezing upon exposure to tone-shock pairings: 64.76% and upon tone-loom pairings: 53.63%; $U=56$, $p=0.65$). The following day both groups of rats were tested for their ability to display learned freezing to the tone cue in a neutral environment. Again, little or no freezing was observed during the baseline period (shock: 0.22% freezing, loom: 0.51% freezing). However, upon the presentation of the tone cue, rats previously exposed to tone-shock pairings during conditioning showed robust freezing that was significantly higher than the low freezing displayed by rats previously exposed to tone-loom pairings (fig. 1C, median increase in shock condition: 94.27%; median increase in loom condition: 5.86%, $U=2$, $p<0.0001$). Although the freezing response to loom-associated tones was low, it was not absent, as the increase in freezing from baseline to the tone was statistically significant (fig. S1B, median increase in freezing: 5.86%, $W=110$, $p=0.0006$). Whether this small increase in freezing reflects a weak associative learning process or the response to the salient tone is unclear. In sum, these results indicate that looming stimuli, while potent drivers of innate freezing, only weakly drive freezing in response to a learned cue.

Looming stimuli are weak drivers of learned escape responses

To test conditioned escape responses, we adapted an existing task where rats display escapes in response to an approaching naturalistic predator (Choi & Kim, 2010). We modified this task to test escapes in response to a visual looming stimulus or to a conditioned tone that was previously paired either with shocks or looms. Rats were first trained during two consecutive days to retrieve food pellets placed at increasing distances; up to 1 meter from a shelter located at one end of a 2-meter runway (fig. 2A). Most rats slowly came out of the shelter and ran back to it as soon as they got the pellet, consuming the pellet inside the shelter (fig. 2B), confirming they regarded the shelter as their “safe space” in the setup.

After the two days of pellet retrieval training, we tested whether looms provoked escape to the shelter in this setup. Rats were tested in the runway with a pellet 1 meter away from the shelter. Once rats reached the middle of the runway, 10 cm before the pellet’s location, a train of looming stimuli was presented on an overhead screen (totaling five 0,5s looms with a 1s interval, fig. 2C). Of the 10 rats tested, one retrieved the pellet before running back to the shelter, with all other 9 rats escaping to the shelter without the pellet (fig. 2D). Of these, 5 rats exited the shelter to retrieve the pellet in the remaining 15 minutes of the experiment (requiring varying times to do so), while the other 4 rats failed to retrieve the pellet (fig. 2E). Interestingly, the rats that took a shorter time to retrieve the pellet were also the ones that required repeated presentation of the looming stimuli to flee to the shelter (fig. 2D and 2E). Hence, both an empty-handed return to the shelter and the amount of time taken to retrieve the pellet seem to be good indicators of the perceived threat level.

Having established the loom-triggered escape task, we turned to studying learned escape responses. We conditioned a new set of rats to a tone cue paired with either shocks or looms, as in the experiment before (except the tone was 1 second long, see Methods and fig. 2A), and tested their response to the tone in the pellet retrieval task. Whereas the lack or low level of freezing responses to a neutral tone has been extensively reported, the response of rats to a neutral yet salient stimulus in this pellet retrieval task was not known, thus, we added a tone-alone control group (see Methods). All of the tone-shock conditioned rats escaped to the shelter before retrieving the pellet (8/8 rats), whereas in the tone-loom and the tone alone groups only 3/7 and 3/6 rats, respectively, did so (fig. 3B). In addition, rats in the tone-shock group took longer to retrieve the pellet than rats in both other groups, which showed similar pellet retrieval times (fig. 3C; we used “pellet survival” curves, which take into account whether and when a pellet was retrieved during the test session; Chi square=11.00, p-value = 0.004). It is possible that the difference seen in escape responses between the tone-shock and tone-loom groups stems from the lower conditioning levels to the tone of the latter group, reflected in lower freezing levels observed during conditioning of these rats (fig. S2). However, none of rats in the tone-shock group retrieved the pellet before retreating to the shelter, regardless of their freezing levels (which varied between 42 and 87%, fig. S2A). In addition, we observed no clear relationship between pellet retrieval time and amount of freezing during conditioning in any of the groups (fig. S2C).

The remaining behaviors, including rearing, reaching, scanning and freezing were scored, but there were no differences in those behaviors across conditions (data not shown). Taken together, these results show that, similar to learned freezing

responses, looming stimuli are not effective at driving robust learned escapes, despite evoking robust innate escape responses.

Discussion

Here we show that a visual looming stimulus, a feature of an approaching predator, induces robust innate defense responses. Using two different setups, we show that if a shelter is available, rats escape to the shelter upon seeing visual looms, while they freeze to looming stimuli if no shelter is present. This indicates that the rats' choice between freezing and fleeing is heavily modulated by context, as previously reported in mice (Vale, Evans, & Branco, 2017; Yilmaz & Meister, 2015). Yet, an auditory cue repeatedly paired with the same looming stimulus failed to trigger these behaviors in a reliable manner, unlike auditory cues paired with shock that drive both responses (fig. 2C and 3C). Multiple factors may explain the discrepancy between the ability of looming stimuli to drive innate responses and the ability to drive these same responses through association with other cues. These stimuli may activate neuronal circuits that are distinct from those controlling the expression of freezing and escapes in responses to learned cues. It is also possible that only close encounters with predators resulting in injury or pain led to a learning process capable of driving acute defensive responses, such as freezing or escape, in response to cues associated with the encounter. Another possibility is that detection of a natural predator involves multiple sensory modalities, and that multisensory integration is important for learning to occur, such that acute responses to an impending learned threat are triggered.

Circuit for innate and learned freezing and escape responses overlap

Although it has been proposed that innate and learned defensive responses rely on partially distinct circuits (Gross & Canteras, 2012), comparison between innate and learned fear studies suggests considerable overlap of involved brain areas. It is well known that the amygdala, especially the lateral (LA), basal (BA) and central (CA) nuclei, are crucial for threat learning and conditioned freezing (Maren, Fanselow, & Angeles, 1995; Tovote et al., 2015; Wilensky, Schafe, Kristensen, & LeDoux, 2006). More recently, these same nuclei of the amygdala have been shown to play a role in innate threat processing and defensive responses, particularly in response to visual looming stimuli (Wei et al. 2015). Information about a visual looming stimulus can reach the amygdala through multiple routes. The superior colliculus, which is well established as a key structure in the processing of visual looms (Billington, Wilkie, Field, & Wann, 2011; Lee, Tran, Turan, & Meister, 2020), indirectly projects to the LA and the CeA, via two separate routes that seem to mediate different response strategies; sustained freezing (Wei et al., 2015) or escape followed by freezing (C. Shang et al., 2015; C. Shang et al., 2018). Strikingly, auditory cues paired with optogenetic activation of the Superior Colliculus (SC) cells in the superficial layer lead to conditioned freezing, indicating that a sufficiently strong activation of the SC could evoke learned freezing responses (C. Shang et al., 2015).

In addition to conditioned freezing, rats also show conditioned escapes in response to cues predicting shocks (but not predicting looms) if given the opportunity. Conditioned escapes have been previously studied using active avoidance paradigms (McAllister, McAllister, & Douglass, 1971). Active avoidance is thought to be

predominantly processed in the LA and BA, and then relayed to the Nucleus Accumbens (Amorapanth, LeDoux, & Nader, 2000; Killcross, Robbins, & Everitt, 1997; Lázaro-Muñoz, LeDoux, & Cain, 2010; Ramirez, Moscarello, Le Doux, & Sears, 2015). Similar to conditioned escapes, inactivating or lesioning the amygdala of rats abolished innate escape and avoidance responses to an artificial robotic predator in a naturalistic foraging task (Choi & Kim, 2010). However, Evans et al., (2018) showed that innate escapes to looming stimuli can bypass the amygdala, revealing a direct connection between the superficial layer of the SC and the dorsal periaqueductal gray (dPAG), where escape responses are thought to be initiated (Ruiz Martinez, Ribeiro De Oliveira, & Brandão, 2006).

In summary, looming stimuli can activate various circuits, most of which include sub-nuclei of amygdala widely implicated in the expression of learned freezing and escape responses. Therefore, we believe that the lack of overlap in neuronal circuitry between innate and learned defensive responses is unlikely to explain the low efficiency of looming stimuli in driving learned freezing or escape.

Pain or injury may be required for threat learning

An alternative explanation for the weak learning we observed to looming associated tones could be the absence of pain and accompanying neuromodulatory responses that are experienced with shocks typically used in studies of conditioned freezing and escapes. Information about pain enters the brain through parallel ascending pathways. One is the spino-thalamic pathway that is connected to the LA through the thalamus, while the second pathway involves projections from the

periaqueductal grey (PAG) to the LA, BA and CeA (Johansen, Tarpley, Ledoux, & Blair, 2010; E. J. Kim et al., 2013). The CeA also receives pain information directly from the Parabrachial nucleus (PBN) (Han et al., 2015). Silencing either the PBN or the PAG during conditioning attenuates conditioned freezing responses (Han et al., 2015; Johansen et al., 2010), while pairing a neutral tone with activation of the PBN or dorsal PAG (dPAG) is sufficient to drive learned freezing to the tone cue in rodents (Han et al., 2015). These findings illustrate the importance of pain information reaching the amygdala in threat learning. Experiencing a painful stimulus evokes the release of neuromodulators such as noradrenaline and acetylcholine, which modulate activity in the amygdala (Galvez, Mesches, & Mcgaugh, 1996; McGaugh, 2000; Naser & Kuner, 2018; Strobel, Hunt, Sullivan, Sun, & Sah, 2014; Wilson & Fadel, 2017). Decreasing or increasing noradrenergic or cholinergic modulation of the amygdala has been shown to attenuate or enhance threat learning, respectively (Bush, Caparosa, Gekker, & LeDoux, 2010; Gu et al., 2020; Jiang et al., 2016). Given that the SC projects directly to the dPAG and the dPAG in turn projects to the locus coeruleus (LC), the main source of noradrenaline to the amygdala, looming stimuli could in theory trigger a noradrenergic response similar to the one triggered by shocks (Ennis, Behbehani, Shipley, van Bockstaele, & Aston-Jones, 1991; Evans et al., 2018; Fallon, Koziell, & Moore, 1978). However, it is currently unknown whether the SC has a functional connection to the LC through the PAG. To examine whether looming stimuli could induce the release of neuromodulators to a similar extent as shocks, more research is required.

Multisensory information may be important for threat learning

Finally, the fact that we did not see learned defensive responses does not necessarily mean that the rats did not learn about the relationship between tone and loom. For example, sensory pre-conditioning studies show that rats can learn to associate two neutral stimuli with each other, but this learning is not accompanied by any overt behavioral response (S. D. Kim, Rivers, Bevins, & Ayres, 1996; Nader & LeDoux, 1999). The same might be true in our study: rats may learn about the association between the tone and the looming stimulus, but not show an acute, overt, defensive response to the tone. Even if a visual looming stimulus is not strong enough to evoke responses after being paired with a tone, it is still possible that the presence of a predator, which constitutes a multimodal set of stimuli, could be more effective at driving learning resulting in acute defensive responses to neutral cues associated to the predator, even in the absence of a painful interaction.

Resume

In conclusion, our work broadens our understanding of learned defensive responses and their boundaries, by showing that the detection of innate cues of threat leads to robust acute defensive responses, but these cues by themselves are not an effective drivers of threat learning. Several requirements may have to be met for a stimulus to be able to drive learned acute defensive responses. An acute defensive response, such as escape or freezing, may require proximity of the threat, while other responses such as avoidance or risk assessment may be displayed in response to more distant threats (Fanselow, 1994). A learned cue, i.e. a stimulus that is associated with the presence of the threat, is necessarily at least one step further from the threat itself.

Thus, it is possible that acute responses are more readily displayed to the direct detection of a threat than to cues learned to be associated with such threat. This implies that for an acute defensive response to be displayed upon the presentation of a learned cue, the learned association between the cue and the threat must be very strong, such as those that result in pain or injury, which in turn depends on the degree of the threat. The dissociation between the capacity to drive an innate acute defensive response and to drive threat learning brings a new perspective to our understanding, both at a mechanistic level of the functioning of survival circuits, and at the behavioral ecology level of the way in which learning from encounters with predators may shape the landscapes of fear.

References

Amorapanth, P., LeDoux, J. E., & Nader, K. (2000). Different lateral amygdala outputs mediate reactions and actions elicited by a fear-arousing stimulus. *Nature Neuroscience*, *3*(1), 74–79. https://doi.org/10.1038/71145

Ball, W., & Tronick, E. (1971). Infant Responses to Impending Collision□: Optical and Real. *American Association for the Advancement of Science*, *171*(3973), 818–820.

Biederman, G. B., & Davey, V. A. (1997). Taste aversion conditioning with heat as an unconditional stimulus: The role of taste intensity and preexposure in rats. *Learning and Motivation*, *28*(1), 140–152. https://doi.org/10.1006/lmot.1997.0959

Billington, J., Wilkie, R. M., Field, D. T., & Wann, J. P. (2011). Neural processing of imminent collision in humans. *Proceedings of the Royal Society B: Biological Sciences*, *278*(1711), 1476–1481. https://doi.org/10.1098/rspb.2010.1895

Blanchard, R. J., Yang, M., Li, C. I., Gervacio, A., & Blanchard, D. C. (2001). Cue and context conditioning of defensive behaviors to cat odor stimuli. *Neuroscience and Biobehavioral Reviews*, *25*(7–8), 587–595. https://doi.org/10.1016/S0149-7634(01)00043-4

Bush, D. E. A., Caparosa, E. M., Gekker, A., & LeDoux, J. (2010). Beta-adrenergic receptors in the lateral nucleus of the amygdala contribute to the acquisition but not the consolidation of auditory fear conditioning. *Frontiers in Behavioral Neuroscience*, *4*(OCT), 1–7. https://doi.org/10.3389/fnbeh.2010.00154

Choi, J. S., & Kim, J. J. (2010). Amygdala regulates risk of predation in rats foraging in a dynamic fear environment. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(50), 21773–21777.

https://doi.org/10.1073/pnas.1010079108

Cruz, A., Heinemans, M., Márquez, C., & Moita, M. A. (2020). Freezing Displayed by Others Is a Learned Cue of Danger Resulting from Co-experiencing Own Freezing and Shock. *Current Biology*, *30*(6), 1128-1135.e6. https://doi.org/10.1016/j.cub.2020.01.025

Dunn, T. W., Gebhardt, C., Naumann, E. A., Riegler, C., Ahrens, M. B., Engert, F., & Del Bene, F. (2016). Neural Circuits Underlying Visually Evoked Escapes in Larval Zebrafish. *Neuron*, *89*(3), 613–628. https://doi.org/10.1016/j.neuron.2015.12.021

Ennis, M., Behbehani, M., Shipley, M. T., van Bockstaele, E. J., & Aston□Jones, G. (1991). Projections from the periaqueductal gray to the rostromedial pericoerulear region and nucleus locus coeruleus: Anatomic and physiologic studies. *Journal of Comparative Neurology*, *306*(3), 480–494. https://doi.org/10.1002/cne.903060311

Evans, D. A., Stempel, A. V., Vale, R., Ruehle, S., Lefler, Y., & Branco, T. (2018). A synaptic threshold mechanism for computing escape decisions, *558*, 590–594. https://doi.org/0.1038/s41586-018-0244-6

Fallon, J. H., Koziell, D. A., & Moore, R. Y. (1978). Catecholamine innervation of the basal forebrain II. Amygdala, suprarhinal cortex and entorhinal cortex. *Journal of Comparative Neurology*, *180*(3), 509–531. https://doi.org/10.1002/cne.901800308

Fanselow, M. S. (1994). Neural organization of the defensive behavior system responsible for fear. *Psychonomic Bulletin & Review*, *1*(4), 429–438. https://doi.org/10.3758/BF03210947

Galvez, R., Mesches, M. H., & Mcgaugh, J. L. (1996). Norepinephrine release in the amygdala in response to footshock stimulation. *Neurobiology of Learning and*

Memory, *66*(3), 253–257. https://doi.org/10.1006/nlme.1996.0067

Gross, C. T., & Canteras, N. S. (2012). The many paths to fear. *Nature Reviews Neuroscience*, *13*(9), 651–658. https://doi.org/10.1038/nrn3301

Gu, Y., Piper, W. T., Branigan, L. A., Vazey, E. M., Aston-Jones, G., Lin, L., … Sears, R. M. (2020). A brainstem-central amygdala circuit underlies defensive responses to learned threats. *Molecular Psychiatry*, *25*(3), 640–654. https://doi.org/10.1038/s41380-019-0599-6

Han, S., Soleiman, M. T., Soden, M. E., Zweifel, L. S., & Palmiter, R. D. (2015). Elucidating an Affective Pain Circuit that Creates a Threat Memory Article Elucidating an Affective Pain Circuit that Creates a Threat Memory. *Cell*, *162*(2), 363–374. https://doi.org/10.1016/j.cell.2015.05.057

Hendersen, R. W., & Graham, J. A. (1979). Avoidance of heat by rats: Effects of thermal context on rapidity of extinction. *Learning and Motivation*, *10*(3), 351–363. https://doi.org/10.1016/0023-9690(79)90038-9

Jiang, L., Kundu, S., Lederman, J. D. D., López-Hernández, G. Y. Y., Ballinger, E. C. C., Wang, S., … Role, L. W. W. (2016). Cholinergic Signaling Controls Conditioned Fear Behaviors and Enhances Plasticity of Cortical-Amygdala Circuits. *Neuron*, *90*(5), 1057–1070. https://doi.org/10.1016/j.neuron.2016.04.028

Johansen, J. P., Tarpley, J. W., Ledoux, J. E., & Blair, H. T. (2010). Neural substrates for expectation-modulated fear learning in the amygdala and periaqueductal gray. *Nature Neuroscience*, *13*(8), 979–986. https://doi.org/10.1038/nn.2594

Killcross, S., Robbins, T. W., & Everitt, B. J. (1997). Different types of fear-conditioned behaviour mediated by separate nuclei within amygdala. *Nature*, *388*(6640), 377–

380. https://doi.org/10.1038/41097

Kim, E. J., Horovitz, O., Pellman, B. A., Tan, L. M., Li, Q., Richter-Levin, G., & Kim, J. J. (2013). Dorsal periaqueductal gray-amygdala pathway conveys both innate and learned fear responses in rats. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(36), 14795–14800. https://doi.org/10.1073/pnas.1310845110

Kim, S. D., Rivers, S., Bevins, R. A., & Ayres, J. J. B. (1996). Conditioned stimulus determinants of conditioned response form in pavlovian fear conditioning. *Journal of Experimental Psychology: Animal Behavior Processes*, *22*(1), 87–104. https://doi.org/10.1037/0097-7403.22.1.87

Kung, J. C., Su, N. M., Fan, R. J., Chai, S. C., & Shyu, B. C. (2003). Contribution of the anterior cingulate cortex to laser-pain conditioning in rats. *Brain Research*, *970*(1–2), 58–72. https://doi.org/10.1016/S0006-8993(02)04276-2

Lázaro-Muñoz, G., LeDoux, J. E., & Cain, C. K. (2010). Sidman Instrumental Avoidance Initially Depends on Lateral and Basal Amygdala and Is Constrained by Central Amygdala-Mediated Pavlovian Processes. *Biological Psychiatry*, *67*(12), 1120–1127. https://doi.org/10.1016/j.biopsych.2009.12.002

LeDoux, J. E. (2000). Emotion Circuits in the Brain. *Annual Review of Neuroscience*, *23*, 155–184.

Lee, K. H., Tran, A., Turan, Z., & Meister, M. (2020). The sifting of visual information in the superior colliculus. *ELife*, *9*, 1–23. https://doi.org/10.7554/eLife.50678

Lopes, G., Bonacchi, N., Frazão, J., Neto, J. P., Atallah, B. V., Soares, S., … Kampff, A. R. (2015). Bonsai: An event-based framework for processing and controlling data

streams. *Frontiers in Neuroinformatics*, *9*(APR), 1–14. https://doi.org/10.3389/fninf.2015.00007

Maren, S., Fanselow, M. S., & Angeles, L. (1995). Synaptic Plasticity in the Basolateral Hippocampal Formation Stimulation Amygdala in viva Induced by, *15*(November).

McAllister, W. R., McAllister, D. E., & Douglass, W. K. (1971). The inverse relationship between shock intensity and shuttle-box avoidance learning in rats: A reinforcement explanation. *Journal of Comparative and Physiological Psychology*, *74*(3), 426–433. https://doi.org/10.1037/h0030579

McGaugh, J. L. (2000). Memory - A century of consolidation. *Science*, *287*(5451), 248–251. https://doi.org/10.1126/science.287.5451.248

Nader, K., & LeDoux, J. E. (1999). Inhibition of the mesoamygdala dopaminergic pathway impairs the retrieval of conditioned fear associations. *Behavioral Neuroscience*, *113*(5), 891–901. https://doi.org/10.1037/0735-7044.113.5.891

Naser, P. V., & Kuner, R. (2018). Molecular, Cellular and Circuit Basis of Cholinergic Modulation of Pain. *Neuroscience*, *387*, 135–148. https://doi.org/10.1016/j.neuroscience.2017.08.049

Oliva, D., Medan, V., & Tomsic, D. (2007). Escape behavior and neuronal responses to looming stimuli in the crab Chasmagnathus granulatus (Decapoda: Grapsidae). *Journal of Experimental Biology*, *210*(5), 865–880. https://doi.org/10.1242/jeb.02707

Pereira, A. G., Cruz, A., Lima, S. Q., & Moita, M. A. (2012). Silence resulting from the cessation of movement signals danger. *Current Biology*, *22*(16), R627–R628. https://doi.org/10.1016/j.cub.2012.06.015

Pereira, A. G., & Moita, M. A. (2016). Is there anybody out there? Neural circuits of threat detection in vertebrates. *Current Opinion in Neurobiology*, *41*, 179–187. https://doi.org/10.1016/j.conb.2016.09.011

Ramirez, F., Moscarello, J. M., Le Doux, J. E., & Sears, R. M. (2015). Active avoidance requires a serial basal amygdala to nucleus accumbens shell circuit. *Journal of Neuroscience*, *35*(8), 3470–3477. https://doi.org/10.1523/JNEUROSCI.1331-14.2015

Ruiz Martinez, R. C., Ribeiro De Oliveira, A., & Brandão, M. L. (2006). Conditioned and unconditioned fear organized in the periaqueductal gray are differentially sensitive to injections of muscimol into amygdaloid nuclei. *Neurobiology of Learning and Memory*, *85*(1), 58–65. https://doi.org/10.1016/j.nlm.2005.08.007

Schiff, W., Caviness, J. A., & Gibson, J. J. (1962). Persistent fear responses in rhesus monkeys to the optical stimulus of “looming.” *Science*, *136*(3520), 982–983. https://doi.org/10.1126/science.136.3520.982

Shang, C., Liu, Z., Chen, Z., Shi, Y., Wang, Q., Liu, S., … Cao, P. (2015). A parvalbumin-positive excitatory visual pathway to trigger fear responses in mice. *Science*, *348*(6242), 1472–1477. https://doi.org/10.1126/science.aaa8694

Shang, Congping, Chen, Z., Liu, A., Li, Y., Zhang, J., Qu, B., … Cao, P. (2018). Divergent midbrain circuits orchestrate escape and freezing responses to looming stimuli in mice. *Nature Communications*, *9*(1), 1–17. https://doi.org/10.1038/s41467-018-03580-7

Strobel, C., Hunt, S., Sullivan, R., Sun, J. Y., & Sah, P. (2014). Emotional regulation of pain: The role of noradrenaline in the amygdala. *Science China Life Sciences*,

57(4), 384–390. https://doi.org/10.1007/s11427-014-4638-x

Takahashi, L. K. (2014). Olfactory systems and neural circuits that modulate predator odor fear. *Frontiers in Behavioral Neuroscience*, *8*(MAR), 1–13. https://doi.org/10.3389/fnbeh.2014.00072

Tovote, P., Fadok, J. P., & Lüthi, A. (2015). Neuronal circuits for fear and anxiety. *Nature Reviews Neuroscience*, *16*(6), 317–331. https://doi.org/10.1038/nrn3945

Usunoff, K. G., Schmitt, O., Itzev, D. E., Rolfs, A., & Wree, A. (2007). Efferent connections of the parabigeminal nucleus to the amygdala and the superior colliculus in the rat: A double-labeling fluorescent retrograde tracing study. *Brain Research*, *1133*(1), 87–91. https://doi.org/10.1016/j.brainres.2006.11.073

Vale, R., Evans, D. A., & Branco, T. (2017). Rapid Spatial Learning Controls Instinctive Defensive Behavior in Mice. *Current Biology*, *27*(9), 1342–1349. https://doi.org/10.1016/j.cub.2017.03.031

Wallace, K. J., & Rosen, J. B. (2000). Predator Odor as an Unconditioned Fear Stimulus in Rats□: Elicitation of Freezing by Trimethylthiazoline, a Component of Fox Feces, *114*(5), 912–922. https://doi.org/10.1037//0735-7044.114.5.912

Wei, P., Liu, N., Zhang, Z., Liu, X., Tang, Y., He, X., … Wang, L. (2015). Processing of visually evoked innate fear by a non-canonical thalamic pathway. *Nature Communications*, *6*, 6756. https://doi.org/10.1038/ncomms7756

Wilensky, A. E., Schafe, G. E., Kristensen, M. P., & LeDoux, J. E. (2006). Rethinking the fear circuit: the central nucleus of the amygdala is required for the acquisition, consolidation, and expression of Pavlovian fear conditioning. *J Neurosci*, *26*(48), 12387–12396. https://doi.org/10.1523/JNEUROSCI.4316-06.2006

Wilson, M. A., & Fadel, J. R. (2017). Cholinergic regulation of fear learning and extinction. *J Neurosci Res.*, *95*(3), 836–852. https://doi.org/10.1002/jnr.23840

Yilmaz, M., & Meister, M. (2015). Report Rapid Innate Defensive Responses of Mice to Looming Visual Stimuli. *Current Biology*, *23*(20), 2011–2015. https://doi.org/10.1016/j.cub.2013.08.015

Zacarias, R., Namiki, S., Card, G. M., Vasconcelos, M. L., & Moita, M. A. (2018). Speed dependent descending control of freezing behavior in Drosophila melanogaster. *Nature Communications*, *9*(1), 1–11. https://doi.org/10.1038/s41467-018-05875-1

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Figures

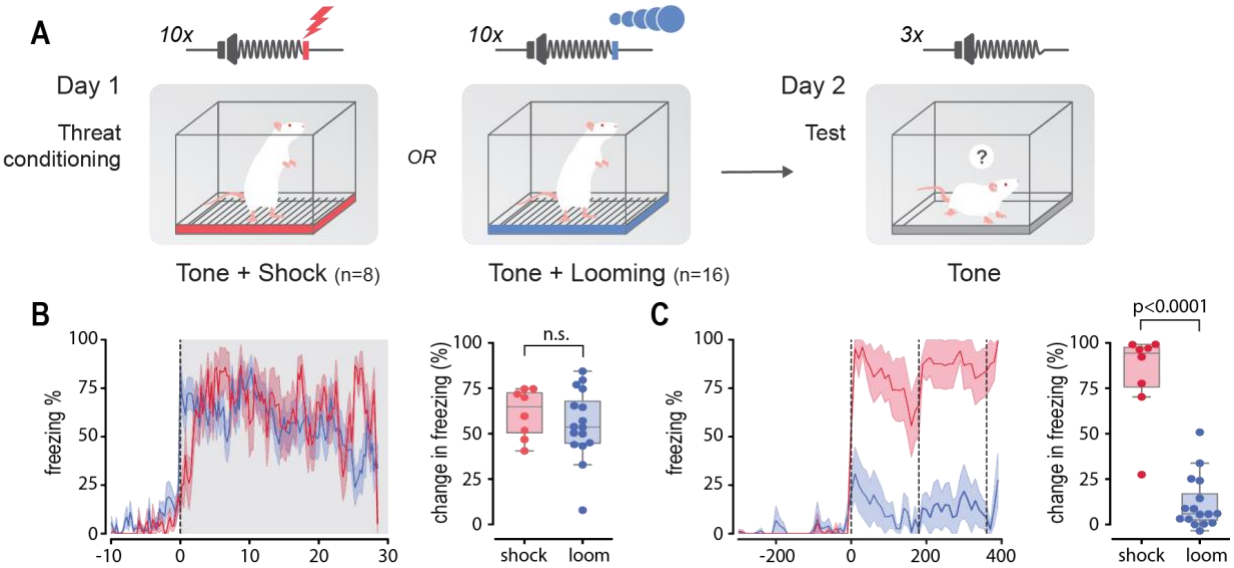


Figure 1 – Rats freeze more to shock-associated tones than loom-associated tones. **(A)** Diagram of behavioral paradigm used to study learned freezing response to conditioned neutral tone. Rats received 10 tone-shock or tone-loom pairings and the next day exposed to 3 tone presentations. **(B)** Freezing during threat conditioning. Left: percentage of time freezing throughout the training session. Baseline period in white (-10 until 0 minutes) and period of exposure to tone-shock/tone-loom pairings in gray (0 until 30 minutes). The line depicts average and the shade represents SEM. Right: change in percentage of time freezing (stimulus presentation period – baseline). Each dot corresponds to an individual animal, the box and whiskers represent the median and interquartile ranges. **(C)** Same as in **(B)** for freezing during the recall test. Gray dotted vertical lines indicate tone delivery. In all panels rats exposed to tone-shock pairings are depicted in red and rats exposed to tone-loom pairings in blue.

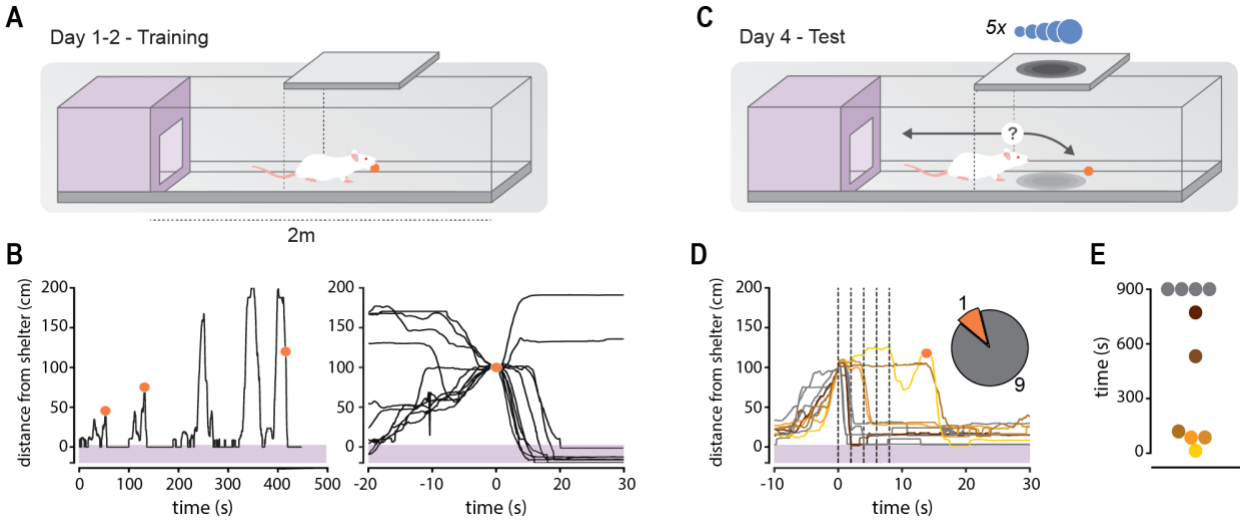


Figure 2 – Escape responses upon looming stimulus presentation during pellet retrieval task.

(A) Diagram of runway used in pellet retrieval training sessions. Rats were trained to retrieve sucrose pellets on two consecutive days, pellets were placed at an increasing distance from the shelter on the left: 0.25, 0.5 and 0.75m from shelter entrance on the first day, 0.5, 0.75 and 1.0m distance on the second day. **(B)** Left: example trace of a rat that retrieved 3 pellets within 500 seconds. The purple shaded area indicates shelter location (-30 to 0 cm) and orange dots indicate when and where a pellet was retrieved. Right: the traces of all animals aligned to retrieval of the last pellet (time of pellet retrieval = 0s). All but two rats went back to the shelter once they retrieved the pellet. **(C)** Diagram of test day. 5 consecutive looms are triggered when rats reached a virtual threshold distance from the shelter (90cm, represented by the dashed line). **(D)** Trajectory of rats aligned to time of loom presentations indicated by vertical dashed lines, starting at t=0s. Most rats returned to the shelter after the first loom, two after the second loom, and only one rat retrieved the pellet before returning to the shelter (orange dot). Inset, pie chart indicating number of pellets retrieved before re-entering the shelter. **(E)** Time elapsed between crossing of loom triggering threshold and pellet retrieval. Each dot corresponds to one animal. Lighter dots represent shorter times for pellet retrieval, while darker colors represent longer retrieval times.

Gray dots represent rats that failed to retrieve the pellet within 15 minutes after loom presentation. The colors of the dots are matched to that of the trajectories of the same animals in **(D)**.

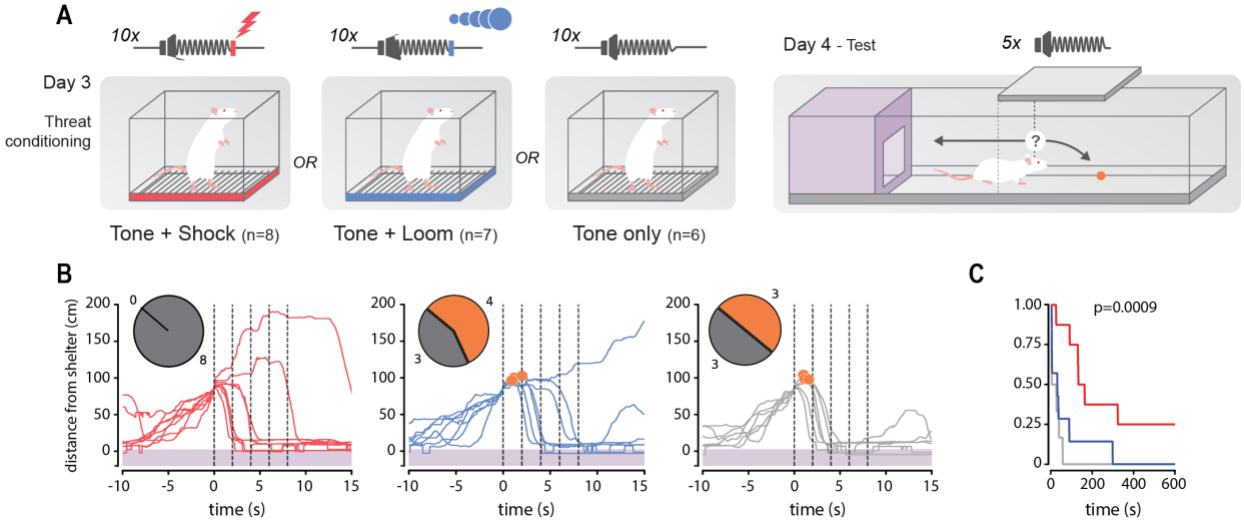
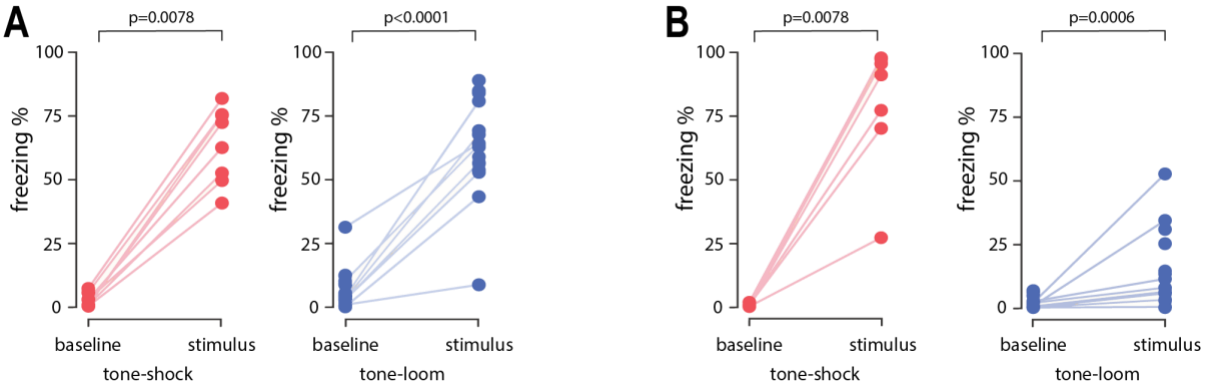


Figure 3 – Escape responses upon conditioned tone presentation during and pellet retrieval task.

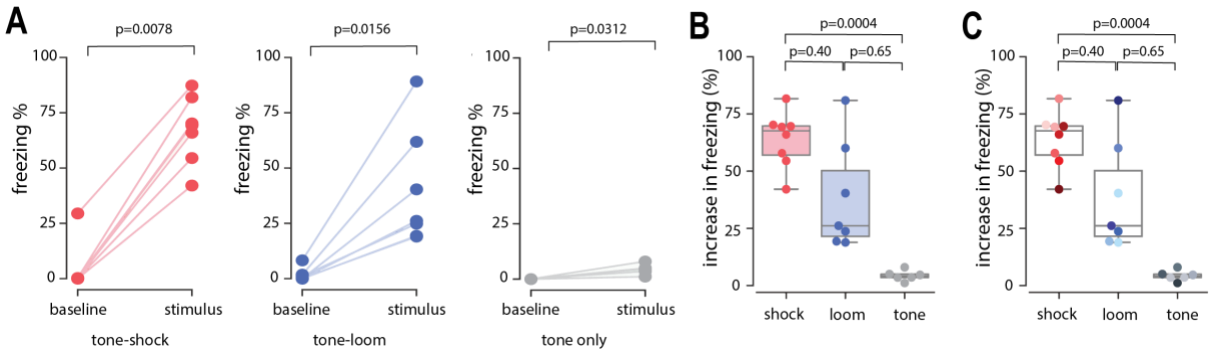
(A) Behavioral paradigm to test conditioned escapes after tone-shock or tone-loom exposure. After two days of training the pellet retrieval task, rats received ten presentations of a neutral tone paired with a shock, a visual loom, or nothing. The next day (day 4), the rats were placed in the runway with a pellet located at 1m distance from the shelter. Crossing the virtual threshold in between the shelter and pellet (dashed line) triggered the presentation of 5 consecutive pure tones previously associated to shock or loom, or neutral. **(B)** Trajectory of rats aligned to time of tone presentations indicated by vertical dashed lines, starting at t=0s. Orange dots indicate when and where a pellet was retrieved. Inset, pie chart indicates number of pellets retrieved before re-entering the shelter. **(C)** "Pellet survival" plots in the different conditions. The p-value represents significance of the differences in survival of the pellets as calculated by a Kaplan-Meier test. The significant difference is driven by the increased survival rates of the pellets in the tone-shock group compared to the two remaining groups. In all panels, rats exposed to tone-shock pairings are depicted in red, rats exposed to tone-loom pairings in blue and rats exposed to tone alone in gray.

Supplemental Figures



Supplemental figure 1 – Percent time freezing per individual during baseline and stimulus period.

(A) Freezing during tone-shock (red) and tone-loom (blue) conditioning; percentage of time spent freezing during baseline and stimulus period per individual. In both conditions the individuals' freezing is low during baseline and significantly increased during the conditioning phase (tone-shock: median increase in freezing: 64.76%, W=36, p=0.0078; tone-loom: median increase in freezing: 53.63%, W=136, p<0.0001). **(B)** Freezing during recall test for tone-shock (red) and tone-loom (blue) conditions; freezing during baseline is low for both conditions and increases during tone presentation. While freezing remains low in the tone-loom condition, the increase per individual is statistically significant in both conditions (tone-shock: median increase in freezing: 96.31%, W=36, p=0.0078; tone-loom: median increase in freezing: 5.76%, W=110, p=0.0006).



Supplemental figure 2 – Individual freezing increase during conditioning.
(A) Freezing of individuals during exposure to tone-shock (red), tone-loom (blue), or tone presentations (gray). While the amount of increase is very low in the tone group, there is a significant increase in freezing in all three conditions (tone-shock: median increase in freezing: 67.62%, W=36, p=0.0078; tone-loom: median increase in freezing: 26.13%, W=28, p=0.0156; tone: median increase in freezing: 4.09%, W=21, p=0.0312). **(B)** Change in freezing per individual for tone-shock (red), tone-loom (blue), and tone (gray) conditions. Freezing increase upon tone exposure is significantly lower than for the tone-shock condition, while tone-loom exposure resulted in an intermediate freezing increase. (Kruskal-Wallis: p<0.0001 (Kruskal-Wallis statistic=14.53). Dunn's multiple comparisons test: shock vs loom: p=0.3998; shock vs tone: p=0.0004; loom vs tone: p= 0.649). **(C)** Same as in **(B)** with individual data points color-coded by pellet retrieval time (lighter shades for faster retrieval), showing no clear relationship between freezing level and time to retrieve the pellet, regardless of condition.