A systematic review of auditory fear conditioning protocols: Animal models and auditory conditioning stimulus

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Abstract. The specific circuits used to investigate fear response have been described on the basis of Pavlovian conditioning or classical fear conditioning procedures. However, prior studies did not utilize identical methods, featuring differences in animal models and exposure tones. This study aimed to identify the most frequently employed animal model and exposure tone that can be feasibly implemented to obtain consistent results in fear conditioning studies. This systematic review analyzed studies on auditory conditioning stimuli effects, i.e., fear conditioning responses, synaptic transmission, synaptic plasticity, long-term potentiation, and associative fear memory, published between 2011 and 2016. Twenty-two studies fulfilled the selection criteria of the analysis. The animal models used were mice (54.5% of studies, with C57BL/6 mice being utilized in 91.7% of the mouse studies) or rats (45.5% of studies, with Sprague–Dawley rats being utilized in 70% of the rat studies). The ranges of loudness, frequency, and duration of tone exposure in the studies were 65–100 dB, 1–12.5 kHz, and 10–30 s, respectively. The most commonly used animal in fear conditioning studies using genetic variation to elucidate certain pathogeneses or therapeutic mechanisms of drugs was the C57BL/6 mouse, whereas Sprague–Dawley rats were frequently used in studies of the physiological responses to auditory fear conditioning, fear memory, synaptic transmission, synaptic plasticity, and associative learning. Significant variation of the tone exposure was also noted.

1. Introduction
Fear is one of the most studied emotions in neuroscience as it influences the survival of organisms. In recent decades, the specific circuits involved in emotional functions have been researched. Most studies have been performed in animals, and they have generally involved Pavlovian conditioning or classical fear conditioning to examine emotional developmental processes, especially fear [1,2]. Classical fear conditioning is performed by providing an unthreatening stimulus (neutral) called the conditioning stimulus (CS), which can be a tone or flashing light, followed by an aversive stimulus, called the unconditioned stimulus (US), such as foot shock. After one or multiple exposures to the combined stimulus, the CS itself can induce fear responses such as defensive behavior (freezing or flight), autonomic nervous system responses (blood pressure and pulse rate changes), and
neuroendocrine responses (stress hormone release). Hence, auditory CS combined with US comprises the auditory fear conditioning (AFC) [1-4].

Pavlovian conditioning has been widely used to investigate the mechanism of fear conditioning responses, synaptic transmission, long-term potentiation (LTP), synaptic plasticity, associative fear memory, and long-term memory [5]. This association between neutral and aversive stimuli is formed and possibly stored in the amygdala. Both human and animal experiments have demonstrated that fear and emotional memories are critically dependent on the amygdala complex. Multiple lines of evidence from electrophysiological, pharmacological, and neurobehavioral studies suggest that synaptic transmission and synaptic plasticity at the sensory inputs to the amygdala play critical roles in the formation and storage of auditory fear memories [1-4]. These studies were conducted under normal conditions and under conditions that disrupt synaptic transmission, associative memory, and learning, such as posttraumatic stress disorder [5-7].

LTP is a cellular model of activity-dependent enhancement of synaptic transmission and a persistent form of synaptic plasticity that is widely studied in models of memory storage in the brain at the synaptic level. In AFC, the auditory stimulus reaches the lateral nucleus of the amygdala (LA) both directly from the auditory thalamus and indirectly via thalamo–cortical and cortical–amygdala connections, and both thalamic and cortical inputs converge on the LA [2,8-10]. LTP can be induced in the LA at both thalamic and cortical auditory inputs in vitro and in vivo [5,11,12].

Various studies have used fear conditioning for different purposes, and these studies employed varied protocols. Interestingly, these studies did not typically use identical methods for AFC, including animal models and exposure tones. Therefore, the present study aimed to identify the most common animal model and exposure tone that can be feasibly implemented to obtain consistent results in fear conditioning studies.

2. Methods
This systematic review analyzed studies on the effects of AFC stimuli, i.e., fear conditioning responses, synaptic transmission, LTP, and associative fear memory. Published studies were retrieved by searching PubMed/Medline using the MeSH terms “Fear Conditioning” OR “Fear Memory” AND “Long term potentiation” NOT “Fear Extinction” over the period 2011–2016. Furthermore, relevant existing articles in our personal library were included.

The inclusion criteria for the articles were as follows: original article, full text including the title written in English, and journal number and publishing institution being included in the primary journal. The studies applied AFC methods in vivo and in vitro to examine synaptic transmission, associative fear memory, long-term memory, synaptic plasticity, LTP, the neuronal circuits involved in the formation of fear conditioning and associative memory, and various disorders. The exclusion criteria were as follows: unfinished or incompletely downloaded article, a focus unrelated to the aim of the review, use of fear conditioning protocols involving a CS other than tones, and use of non-animal models. The data collected from these articles and journals were grouped according to the following categories: study aim, tone characteristics, and animal model. The outcomes of the studies were noted, tabulated, and synthesized. The various animal models and tone characteristics were compared according to the study outcome in terms of synaptic transmission, LTP, and associative fear memory for analysis.

3. Results
In this study, 50 articles were retrieved (32 from the database search on November 23, 2016 and 18 from our personal library). However, only 22 articles met the inclusion criteria [13-34]. Fourteen articles did not meet the inclusion criteria, whereas other articles were excluded as follows: human subjects, four articles; failure to use an auditory stimulus as CS, nine articles; and incomplete download, one article.
4. Discussion
A comparison of the aims, tone characteristics, animal models, and outcomes of the 22 included studies is presented in Table 1.

**Table 1.** Comparison of the aims, tone characteristics, animal models, and outcomes of the reviewed studies

| Ref | Study aim                                                                 | Tone                        | Animal model                                                  | Study outcome                                                                 |
|-----|---------------------------------------------------------------------------|-----------------------------|---------------------------------------------------------------|------------------------------------------------------------------------------|
| [13] | Elucidated the pathogenesis of schizophrenia-like symptoms                | 90 dB, white noise, 30 s    | Schizophrenia model: male Long Evans rats (250–300 g)         | Synaptic transmission, LTP in the amygdala and hippocampus, and the processing of fear-related memory were impaired |
| [14] | Elucidated the pathogenesis of Angelman syndrome                          | White noise (ventilation fans), 30 s | Angelman syndrome model: C57BL/6 mice (10–16 weeks old)      | LTP in the hippocampus and long-term contextual fear memory were impaired   |
| [15] | Elucidated the pathogenesis of Coffin–Lowry syndrome                      | 80 dB, 1 kHz, 15 s          | Coffin–Lowry model: C57BL/6J mice (3–7 months old)           | Synaptic transmission and LTP in the hippocampus were impaired               |
| [16] | Elaborated the physiology of fear acquisition, fear response, fear memory, synaptic transmission, and synaptic plasticity | 85 dB, 2.8 kHz, 30 s       | Male Sprague–Dawley rats (3–5 weeks old)                     | LTP and associative fear learning were induced                               |
| [17] | Elucidated the pathogenesis of amyotrophic lateral sclerosis and Parkinson’s disease | 75 dB, 4 kHz, 30 s         | 129/SvEv mice crossed with C57BL/6 mice                     | Enhanced LTP in the dentate gyrus                                           |
|      |                                                                           |                             |                                                              | Normal cued fear conditioning                                                |
|      |                                                                           |                             |                                                              | Deficit in contextual fear conditioning                                      |
| [18] | Elaborated the physiology of fear acquisition, fear response, fear memory, synaptic transmission, and synaptic plasticity | 96 dB, 2.2 kHz, 10 s      | Male Sprague–Dawley rats (180–200 g)                        | AFC induced synaptic transmission in the cortico–amygdala pathway and enhanced the long-term retention of fear memory |
|      |                                                                           |                             |                                                              | AFC decreased LTP induction in the cortico–amygdala pathway, thus impairing long-term synaptic plasticity |
| [19] | Elaborated the physiology of fear acquisition, fear response, fear memory, synaptic transmission, and synaptic plasticity | 80 dB, white noise, 15 s  | Adult male F344 rats                                         | LTP in the hippocampus and intrinsic neuronal excitability in the CA1 hippocampal region were enhanced |
| [20] | Elucidated the pathogenesis of neurocognitive disorders                   | 100 dB, 2.8 kHz, 30 s      | Male C57/BL6 mice (6–10 weeks old)                           | UE2316 prevented the suppression of synaptic potentiation, which is correlated with plasma corticosterone levels, and impaired contextual, but not cued, fear conditioning for the freezing response |
### Table 1. Continue

| Ref  | Study aim                                                                                   | Tone                   | Animal model                                      | Study outcome                                                                                     |
|------|---------------------------------------------------------------------------------------------|------------------------|--------------------------------------------------|---------------------------------------------------------------------------------------------------|
| [21] | Elaborated the physiology of fear acquisition, fear response, fear memory, synaptic transmission, and synaptic plasticity | 85 dB, 2.8 kHz, 30 s   | Male Sprague–Dawley rats (4–5 weeks old)          | Fear conditioning occluded L-LTP at T-LA synapses                                                  |
| [22] | Elaborated the physiology of fear acquisition, fear response, fear memory, synaptic transmission, and synaptic plasticity | 65 dB, 29 s            | Male Sprague–Dawley rats (200–250 g)             | CGRP supported synaptic plasticity in the CeA by inducing LTP in the BLA-CeA pathway via an NMDAR-dependent LTP mechanism |
| [23] | Elucidated pathogenesis of panic disorder and amygdala dysfunction                          | 80 dB, 20 s            | C57BL/6 mice (1–5 months old)                    | LTP in the amygdala and fear learning were impaired in ASIC1a-null mice                          |
| [24] | Elaborated the physiology of fear acquisition, fear response, fear memory, synaptic transmission, and synaptic plasticity | 85 dB, white noise, 30 s | Mice with a floxed Fzr1 allele                   | LTP in the amygdala and associative fear memory were impaired in Cdh1 cKO mice                  |
| [25] | Elucidated the pathogenesis of the pathological condition in restless legs syndrome         | 90 dB, 30 s            | C57BL/6J mice                                    | Cued and contextual fear memory for freezing responses were enhanced in Btbd9-mutant mice       |
| [26] | Examined the effects of the dietary PUFA ratio on fear acquisition and fear memory consolidation | 70 dB, 10 kHz, 30 s | Male C57BL/6 mice (4–5 weeks old)                | A high ω3 to ω6 PUFA ratio diet reduced fear responses and fear memory in AFC                   |
| [27] | Elucidated the pathogenesis and treatment of neurocognitive disorders                       | 90 dB, 5 kHz, 30 s    | Male C57BL/6 mice (24–28 g, 6–8 weeks old)      | DG-GSK3β-silenced mice exhibited impaired contextual fear memory formation                        |
| [28] | Elaborated the physiology of fear acquisition, fear response, fear memory, synaptic transmission, and synaptic plasticity | 70 dB, 8 kHz, 15 s    | C57BL/6J mice (4–6 weeks old)                    | CASKO: Long-term potentiation and remote contextual fear memory were impaired                    |
| [29] | Elaborated the physiology of fear acquisition, fear response, fear memory, synaptic transmission, and synaptic plasticity | 80 dB, 1 kHz, 15 s    | Wistar rats (250–300 g)                          | PKMζ-overexpressing slices displayed enhanced long-term memory for contextual, but not cued, fear memory |
| [30] | Elaborated the physiology of fear acquisition, fear response, fear memory, synaptic transmission, and synaptic plasticity | Sine wave, 80 dB, 9 kHz, 20 s | Male C57BL/6N mice (3–5 months old) | CBI KO mice displayed an enhanced freezing level in contextual fear conditioning                  |
### Table 1. Continue

| Ref | Study aim                                                                                                                                                                                                 | Tone | Animal model                                                                 | Study outcome                                                                                                                                                                                                 |
|-----|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| [31] | Studied the sex-related susceptibility and the role of testosterone in fear memory                                                                                                                       | 75 dB, 10 s | Male and female C57BL/6 mice (8–9 weeks old)                                   | Testosterone decreased LA LTP and cued fear memory; ovarian hormones and LA LTP were loosely associated with cued fear memory                                                                                                                                 |
| [32] | Investigated the role of NO in the physiology of fear acquisition and fear memory consolidation                                                                                                             | 85 dB, 2.8 kHz, 30 s | Male Sprague–Dawley rats                                                      | NO suppressed pre-LTP in the cortical input but promoted post-LTP in the thalamic input, both of which contributed to encoding conditioned fear memory                                                                 |
| [33] | Elaborated the physiology of fear acquisition, fear response, fear memory, synaptic transmission, and synaptic plasticity                                                                                  | 77 dB, 4 kHz, 30 s | Male Sprague–Dawley rats (3–6 weeks old)                                      | Fear expression is modulated by inputs from pre-limbic cortex and ventral hippocampus                                                                                                                                 |
| [34] | Elaborated the physiology of fear acquisition, fear response, fear memory, synaptic transmission, and synaptic plasticity                                                                                | Pure tone (2.8 kHz, 80 dB), white noise (80 db), or FM tone (12.5 kHz carrier frequency, 50 Hz modulation frequency, 2.5 kHz modulation depth, 1 ms ramp, 80 dB), for 30 s | Male Sprague–Dawley rats (4–6 weeks old)                                      | A fear conditioning response emerged during AFC                                                                                                                                                                |

Pre-synapse LTP in T-LA did not occur in any group, whereas C-LA occurred only in the white noise group

**Ref:** reference number; **UE2316:** selective 11βHSD1 inhibitor; **L-LTP:** late-phase long-term potentiation; **T-LA:** thalamo–lateral amygdala; **CGRP:** calcitonin gene-related peptide; **CeA:** central nucleus of the amygdala; **BLA:** basolateral nucleus of the amygdala; **NMDAR:** N-methyl-D-aspartate receptor; **ASIC1a:** acid-sensing ion channel-1a; **Cdh1:** E-cadherin gene; **Cdh1 cKO:** Cdh1 conditional knockout; **BTBD9:** BTB (POZ) domain containing 9 gene; **PUFA:** polyunsaturated fatty acid; **DG:** dentate gyrus; **GSK3β:** glycogen synthase kinase-3 beta; **CASKO:** CaMKIIα-Cre–induced knockout of Shp2; **CaMKII:** Ca²⁺/calmodulin-dependent protein kinase II; **PKMζ:** protein kinase Mζ; **CB1-KO:** cannabinoid type 1 knockout; **FM:** frequency modulated; **C-LA:** cortico–lateral amygdala; **ITDP:** input-timing-dependent plasticity; **NO:** nitric oxide

#### 4.1. Comparisons of fear conditioning implementation between studies

Eight studies used fear conditioning procedures to elaborate the physiology of fear acquisition, fear response, fear memory, synaptic transmission, synaptic plasticity, associative learning, and memory based on Pavlovian studies. The proteins and genes underlying learning and memory processes were investigated [16,18,19,21,22,24,28-30,33,34]. Eight other studies elucidated the pathogenesis and/or treatment of diseases or symptoms and correlated the findings with fear conditioning and fear responses to confirm the results [13-15,17,20,23,25,27]. The influence of dietary ratios (polyunsaturated fatty acids), chemical substances (nitric oxide), and hormones (testosterone and...
ovarian hormones), as well as of sex-related variables, on the physiology of fear acquisition and fear memory consolidation has also been investigated [26,31,32].

Studies of neurocognitive disorders for which the amygdala and/or hippocampus are the neuroanatomical sites of origin have used fear conditioning to explain the pathogenesis and confirm the outcome of fear responses. The amygdala is responsible for the storage of cued fear memory. Conversely, the hippocampus is responsible for the storage of contextual memory. Fear conditioning is used as confirmation by observing the duration of freezing responses related to cued and/or context stimuli in animal models. However, the methods and outcomes of these studies were not identical (Table 1). Therefore, to clarify the effects of fear conditioning procedures, similar outcome measures must be employed. Tables 2–3 present a comparison of the tone characteristics and animal models of various studies 2.

Table 2. Comparison of the animal models in the review study

| Animal model | Strain       | Total | Presentation |
|--------------|--------------|-------|--------------|
| Rat          | Sprague–Dawley | 7     | 70%          |
|              | Long Evans   | 1     | 10%          |
|              | Wistar       | 1     | 10%          |
|              | F344         | 1     | 10%          |
| Mouse        | C5BL/6       | 11    | 91.7%        |
|              | Others       | 1     | 8.3%         |

Table 3. Comparison of tone characteristics and animal models in the reviewed studies

|              | Median | Mean | Range |
|--------------|--------|------|-------|
| Weight (g)   | Rats   | 225  | 275   | 220 | 263  | 180 | 300 |
|              | Mice   | -    | -     | -   | -    | 24  | 28  |
| Age (weeks)  | Rats   | 3.5  | 5.5   | 3.5 | 5.5  | 3   | 6   |
|              | Mice   | 6    | 10    | 7.3 | 13.6 | 4   | 28  |
| Intensity (dB)| Rats | 82.5 | 82.3  | 65  | 96   |
|              | Mice   | 80   | 81.4  | 70  | 100  |
| Frequency (kHz)| Rats | 2.8  | 3.8   | 1   | 4    |
|              | Mice   | 5    | 5.7   | 1   | 12.5 |
| Duration (s) | Rats   | 30   | 24.9  | 10  | 30   |
|              | Mice   | 30   | 24.2  | 10  | 30   |

4.2. Animal model comparison
As noted in Tables 1–2, most studies used various strains of mice and rats as animal models. In total, 54.5% of these studies used mice, and 91.7% of the mouse studies used C57BL/6 mice. Rats were used in the remaining 45.5% of these studies, and most rat studies (70%) employed Sprague–Dawley rats. The selection of the animal model may have been related to the general aim of each study, such as studying the pathogenesis of certain diseases or investigating the therapeutic mechanisms of drugs.

C57BL/6 mice are used as a general-purpose strain and as a background strain for the generation of congenic mice carrying both spontaneous and induced mutations. It has a permissive background for the maximal expression of most mutations. Later in life, these mice can develop severe and progressive age-related hearing-loss, including the disruption of both outer and inner hair cells, and they are more susceptible to noise-induced hearing loss, making them useful for sensorineural research
On the other hand, Sprague–Dawley rats have been used widely as a general-multipurpose model for safety and efficacy testing, and as models for studying aging, nutrition, diet-induced obesity, and oncogenesis [36]. Research in which gene mutations were introduced to investigate the pathogenesis of various diseases or therapeutic mechanisms of drugs used C57BL/6 mice instead of rats. Conversely, studies that investigated the physiology of fear conditioning responses, fear memory, synaptic transmission, LTP, and associative fear memory used either rats or mice.

As illustrated in Table 3, the studies used mice and rats in the postnatal period, but the weights and ages of the animals at the time at which the fear conditioning protocol was conducted differed. The mice used in these studies ranged in age from 4 to 28 weeks, with rats having an age range of 3–6 weeks. The weights of the rats in these studies ranged from 180 to 300 g, whereas only one mouse study reported a weight range of 24–28 g [27].

In general, laboratory rats reach puberty when skeletal growth tapers, which occurs at 39–47 days of age in males and at 34–38 days in females. The animals reach maturity at 5–6 months of age. In the weaning period, laboratory rats should weigh approximately 30–55 g versus around 150–200 g in puberty. Mice reach sexual maturity at approximately 4 weeks of age and enter the middle age at 10 months. Regarding sexually mature mice, the weights of females and males should exceed 14.1 and 15.7 g, respectively [37-39]. Hence, the mouse studies used sexually mature animals based on their ages and weights. Contrarily, the ages of the rats in the reviewed studies indicated that the animals’ maturity ranged from weaning to full sexual maturity, whereas the maturity ranged from puberty to full sexual maturity based on weight.

As laboratory rats are used as animal models, it is important to know their life span. Short-lived rats are used to investigate certain diseases, whereas long-lived rats are used to study the mechanism of aging [40]. Thus, as fear conditioning protocols are employed to assess fear conditioning responses, synaptic transmission, and associative fear memory, it is probably preferable to use short-lived animals to avoid issues related to progressive age-related hearing loss. If the study is examining the expression of spontaneous or induced mutations, it is probably desirable to use C57BL/6 mice; however, either rats or mice can be used to investigate the physiological response of auditory fear conditioning.

4.3. Auditory CS
The intensity of the auditory stimuli used in these studies surpassed the known threshold distribution of most auditory thalamus neurons, which relay auditory information to the LA. Laboratory rats can hear frequencies of 500 Hz to 60–80 kHz, even if the decibel level does not exceed that of background noise (80–95 dB). Unlike rats, mice can hear frequencies of 2.3–85.5 kHz. Although rats can hear sounds unperceivable to humans, they experience damage at similar decibel levels as humans, namely mechanical damage at 160 dB, pain at approximately 140 dB, and signs of inner ear damage after prolonged exposure to approximately 100 dB [41,42]. However, the studies used different tones, including studies involving the same animal strain (Tables 1 and 3). The tones used as CS differed in frequency, intensity, and duration of exposure. These discrepancies were due to differences in the method of fear conditioning used in these studies such as cued, contextual, delayed, or trace fear conditioning. In these studies, the ranges of loudness, frequency, and duration of the tone exposure were 65–100 dB, 1–12.5 kHz, and 10–30 s, respectively.

Most studies used previously reported protocols without considering whether different tones have dissimilar implications regarding the outcome of fear acquisition and fear memory processes. A recent study revealed that different characteristics of tones as CS superficially elicited similar responses and fear memories, but their molecular and cellular mechanisms were distinct, for example, in the form of LA synaptic plasticity, which led to different fear memories for each tone [34]. Hence, the most optimal tone for any particular purpose remains elusive.
Therefore, to obtain consistent results when investigating synaptic transmission, synaptic plasticity, and associative memory using fear conditioning, a standardized protocol is needed, and research is needed to clarify the proper animal model and protocol for this purpose.

4.4. Suggestions for future research
As various methods have been used for fear conditioning, it is necessary to standardize the animal model as well as the tone used as the auditory CS. The effects of the strain, age, and weight of the model animal on outcomes related to synaptic transmission, synaptic plasticity, and formation of fear memory must be considered. We have suggested the use of short-lived animal models, but care should be taken to avoid the use of older animals due to the potential effects of progressive age-related hearing loss.

Further investigation is also needed regarding tone exposure. Although different tones as CS superficially elicited similar responses and fear memories, their molecular mechanisms may be distinct; therefore, further questions have emerged such as whether certain tones enhance the effects of fear conditioning and whether different tones are associated with distinct forms of LTP and other molecular bases of synaptic plasticity and transmission.

5. Conclusion
In conclusion, fear conditioning is widely used to clarify the mechanisms of associative memory formation, synaptic transmission, and plasticity in various studies. However, the method used for fear conditioning has not been standardized. In these studies, C57BL/6 mice were most commonly used in studies utilizing genetic mutations to elucidate the pathogenesis of diseases or therapeutic mechanisms of drugs, whereas Sprague–Dawley rats were frequently used in studies of the physiological responses, the cellular or molecular basis of auditory fear conditioning, fear responses, fear memory, synaptic transmission, synaptic plasticity, and associative learning. Wide variation was also noted concerning the characteristics of the exposure tone. Hence, to obtain consistent results, more studies are needed to identify the optimal animal model and tone used for fear conditioning.

Conflict of interest
I confirm that this article has no conflicts of interest.

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