• Review •

Using the conditioned fear stress (CFS) animal model to understand the neurobiological mechanisms and pharmacological treatment of anxiety

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Summary: The mechanisms underlying the etiology and pathophysiology of anxiety disorders — the most prevalent class of mental disorders — remain unclear. Over the last 30 years investigators have used the animal model of conditioned fear stress (CFS) to investigate the brain structures and neurotransmitter systems involved in aversive emotional learning and memory. Recent studies have focused on the neuronal circuitry and cellular mechanisms of fearful emotional experiences. This review describes the CFS paradigm, discusses the neural circuit and neurotransmission underlying CFS, and explains the mechanism of action of pharmacological treatments of CFS. The focus of the review is on the molecular mechanisms of fear extinction, a phenomenon directly implicated in the clinical treatment of anxiety. Based on our assessment of previous work we will conclude by considering potential molecular targets for treating symptoms of anxiety and fear.

1. Introduction

Anxiety disorders are the most common type of mental disorder; the reported lifetime prevalence is up to 14%.[1] Benzodiazepines are effective in the treatment of anxiety disorders, but adverse effects — particularly the dependence that may occur with prolonged use at high doses — limit their clinical application. Starting in the 1980s, selective serotonin reuptake inhibitors (SSRI), which were primarily used for depression, began to play an increasingly important role in the treatment of anxiety disorders. SSRIs increase concentrations of 5-HT in the synapse cleft and thus have anxiolytic effects for almost all subtypes of anxiety disorders, including general anxiety disorder, panic disorder, social anxiety, and obsessive-compulsive disorder. In recent years, 5-HT1A agonists such as tandospirone and buspirone have also been proven to be effective for alleviating anxiety, suggesting that the regulation of anxiety and fear states involves both serotonergic systems as well GABAergic systems. But there remain many gaps in our understanding of the underlying mechanisms.

Using the learning theory framework, anxiety or fear can be considered an emotional learning process that includes the acquisition, consolidation, expression (retrieval) and extinction of aversive emotional memories. Support for this approach comes from several studies which find that glutamatergic systems are associated with emotional learning and memory. This review describes the conditioned fear stress (CFS) paradigm, an animal model used to study anxiety and fear, and reviews the neural circuits and neurotransmission processes underlying the CFS, highlighting the mechanisms of action of pharmacological treatments. Finally, we discuss future directions in the study of the neurobiological mechanisms of anxiety and fear, and suggest some new approaches to identifying pharmacological treatment targets for anxiety disorders.

2. Conditioned fear stress

2.1 Behavioral procedure

Pavlovian fear conditioning is one of the most extensively studied and reliable behavioral paradigms for understanding the mechanisms involved in fear and anxiety. In this paradigm, neutral conditioned stimuli (such as a tone, a light or environmental context) are paired with aversive unconditioned stimuli (such as an electric shock) that reflexively evoke an unconditioned fear response. According to Shumyatsky and colleagues,[2] there are two types of conditioned stimuli, one unimodal, the other multimodal. A unimodal conditioned stimulus refers to a discrete cue affecting

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a single sensory modality such as a tone, a light, or an odor. In contrast, a multimodal conditioned stimulus influences multiple sensory modalities, such as a specific physical setting or context.

After acquiring the association of conditioned and unconditioned stimuli, the conditioned stimulus is gradually capable of inducing a conditioned response, such as fear-related behaviors (freezing, being startled), and the associated physiological, biological and neuroendocrine changes.[3] Subsequent repeated exposure to the conditioned stimulus in the absence of the aversive event results in the decline and eventual extinction of the conditioned response, a process that appears to be the result of new learning rather than simple forgetting of the conditioned fear. In conditioned fear stress (CFS), experimental manipulations involving drugs, brain lesions and so forth are made at different time points during and after the learning process to determine their effect on the acquisition, consolidation and extinction of conditioned fear. Recent studies using the CFS paradigm have focused on the process of extinction because of its presumed link to the effective treatment of anxiety disorders.[4,5]

2.2 Neural circuits in CFS

Several brain regions, particularly the amygdala and hippocampus, have been implicated in fear conditioning. The amygdala is the most important conduit for neural circuits involved in Pavlovian conditioning. It is the central brain region where conditioned stimuli and unconditioned stimuli converge and thus has a key role as the sensor-motor interface for fear.[5-9] Anatomically, the amygdala consists of several distinct nuclei with different functions, including the lateral, basolateral, basomedial and central amygdaloid nuclei.[9,10] The lateral, basolateral and basomedial nuclei are collectively referred to as the basolateral complex; this complex is the primary sensory interface of the amygdala and thus the probable site within the amygdala for establishing the association between the conditioned and unconditioned stimuli. Confirming this coordinating role of the basolateral complex, many studies find that selective lesions of the complex induces profound deficits in both the acquisition and expression of fear conditioning.[11-13] The central amygdaloid nucleus receives afferents projection from the basolateral complex and has projections to the hypothalamus and brainstem; these connections suggest that it is the final common pathway for the fear responses. Lesions of the central amygdaloid nucleus also produce severe deficits in both the acquisition and expression of conditional fear.[14-16]

The hippocampus is another important site involved in fear conditioning. It participates in the acquisition of fear by providing contextual (or spatial) memory of the conditioned stimulus to the amygdala.[17,18] That is, the hippocampus is responsible for assembling contextual representations of the conditioned stimulus and conveying these representations to the amygdala for association with the unconditioned stimulus. Studies showing that electrolytic lesions of the dorsal hippocampus can prevent both the acquisition and expression of contextual fear conditioning confirm this role of the hippocampus.[17,19] The hippocampus also plays an important role in the consolidation, storage and retrieval of fear-related memory.[20] Lesions of the hippocampus made shortly after conditioning is completed generate a profound retrograde amnesia for contextual fear[17,21] — human amnesia also occurs if there is injury to the hippocampus soon after learning.[22]

Several other brain regions have been implicated in fear conditioning. a) Attention has been focused on the role of the prefrontal cortex because some studies report that lesions in this region impair the extinction of fear.[23,24] However, other studies have not confirmed these results[25] so no clear conclusions are yet possible. b) Lesions in the ventrolateral column of the periaqueductal gray region either before or after conditioning abolished stress-induced freezing behaviors.[26] And c) lesions of the mediodorsal thalamic nucleus before or after training are also associated with attenuated stress-induced freezing behaviors.[27]

2.3 Neurotransmission in CFS

2.3.1 Serotonergic systems

An increasing body of evidence confirms the role of serotonergic systems in the regulation of anxiety and fear states. Dysregulation of serotonergic systems has been linked to anxiety, depression and other stress-related mental disorders. An early report by Klein[28] in 1964 demonstrated that the tricyclic antidepressant imipramine was effective in the treatment of panic disorder. And since the 1990s, SSRIs (which facilitate serotonin neurotransmission by increasing extracellular serotonin concentration)[29] have been widely used to treat most anxiety disorders and are now the first-line treatment for these disorders in many locations.[30] But despite the well-established clinical evidence demonstrating that drugs acting on serotonergic systems have anxiolytic action, the exact mechanisms of action, the specific subtype of 5-HT receptor that is responsible, and the location in the brain that is involved remain unclear.

CFS is a useful animal model of anxiety that can help to resolve some of these questions. Several studies using the CFS paradigm have demonstrated that fear conditioning increased serotonin neurotransmission in the medial prefrontal cortex and amygdala.[31-33] CFS also induced c-Fos expression in the basolateral complex, locus coeruleus, and dorsal raphe nucleus,[31,34] and treatment with citalopram attenuated contextual CFS-induced c-Fos expression in the basolateral complex.[35] Fear conditioning decreased the firing fre-

quency of pyramidal neurons in the CA1 region of the hippocampal, but the administration of a 5-HT1A antagonist blocked this decrease in firing frequency.[36] Using mice with central deficiencies in serotonin, Dai and colleagues[37] found that the 5-HT deficiency enhanced contextual fear learning and memory, an effect that was reversed with intracerebroventricular administration of 5-HT; administration of 5-HT to the 5-HT-deficient mice also restored alterations of hippocampal synaptic plasticity induced by electric shocks. Monoamine oxidase (MAO) inhibitors reduced conditioned fear, but the effect was only seen when both isoforms (MAO-A and MAO-B) were used.[38] Citalopram, administrated either by systemic injection or as a microinjection to the amygdala, reduced stress-related freezing behavior.[39,40] Fluoxetine also reduced freezing behaviors and restored the startle response, but the effect was only evident when given intense (not moderate) fear conditioning.[41] Fluvoxamine and milnacipran (a serotonin noradrenaline reuptake inhibitor) also suppress CFS-induced freezing.[42] Interestingly, the α1 adrenoreceptor antagonist prazosin attenuated the citalorpram or mirtazapine-induced decrease in conditioned freezing, suggesting that the noradrenergic system has an effect on the serotonin levels in fear conditioning.[43,44] Mirtazapine, a noradrenergic and specific serotonergic antidepressant (NaSSA), has also been shown to have anxiolytic effects in CFS.[44]

Apart from the 5-HT transporter, the 5-HT1A receptor is another potential target in the treatment of anxiety disorders.[45] 5-HT1A receptor agonists such as buspirone and tandospirone have anxiolytic action in CFS models.[38,46,47] Anatomic studies show that both the amygdala and the hippocampus receive serotonergic innervation from the dorsal raphe nucleus[48,49] and have several subtypes of serotonin receptors, including the 5-HT1A receptor. Studies using the CFS paradigm have shown that 5-HT1A receptor agonists such as ipsapirone, flesinoxan, 8-OH-DPAT, and others can attenuate conditioned freezing.[50-52] It has also been shown that the 5-HT1A agonist flesinoxan inhibits fear conditioning through stimulation of postsynaptic 5-HT1A receptors in the amygdala and hippocampus.[53] Stiedl and colleagues[54] microinjected another 5-HT1A receptor agonist, 8-OH-DPAT, into the hippocampus of mice exposed to CFS and found reduced acquisition of fear conditioning; similarly, infusions of 8-OH-DPAT into the median raphe nucleus and dorsal hippocampus reduced contextual conditioned freezing.[55] Coadministration of the 5-HT1A agonist tandospirone with paroxetine, fluvoxamine or citalopram inhibited conditioned freezing, suggesting a synergistic effect of 5-HT1A agonists and SSRIs. This result is similar to previous work by Li[56] which found augmented anxiolytic effects by combining the 5-HT1A agonist flesinoxan with the SSRI fluvoxamine. Taken together, the results suggest that adjunctive treatment with 5-HT1A receptor agonists in persons with anxiety disorders being treated with SSRIs could result in improved clinical outcomes.

McDevitt and colleagues[57] found that 5-HT1B overexpression in the caudal dorsal raphe nucleus reduced expression of conditioned fear and depression-like behavior; they also found that systemic administration of the 5-HT1B agonist CP-94,253 reduced freezing. Muraki[58] assessed the effects of administration of a selective 5-HT1A receptor antagonist (WAY 100,635) and a selective 5-HT1B/1D receptor antagonist (GR 127, 935) on the anxiolytic effect of citalorpram in CFS: coadministration of the 5-HT1A antagonist enhanced the anxiolytic effect of citalopram by facilitating central 5-HT neurotransmission but coadministration of the 5-HT1B/1D receptor antagonist did not change the magnitude of the anxiolytic effect of citalopram.

Taken together these studies show that drugs or agents that increase extracellular 5-HT concentration (MAO inhibitors, SSRIs, SNRIs and NaSSAs) and those that agonize the 5-HT1A or 5-HT1B receptors (5-HT1A or 5-HT1B agonists) in the amygdala or hippocampus can reduce conditioned fear. These findings give strong support for the suggestion that serotonergic systems are closely associated with fear conditioning.

2.3.2 N-methyl-D-aspartate (NMDA) and long-term potentiation

A growing body of evidence suggests that the cellular mechanism underlying fear conditioning is long-term potentiation by N-methyl-D-aspartate (NMDA) receptors in both the hippocampus and amygdala.[59-62] Fear conditioning has been shown to stimulate long-term potentiation in the amygdala[63,64] and contextual fear conditioning is correlated with long-term potentiation in the hippocampus.[65-67] The notion that involvement of both the hippocampal and amygdaloid NMDA receptors in fear conditioning is supported by several studies which report that intra-amygdala or hippocampal infusions of the NMDA receptor antagonists APV or MK-801 effectively block both the acquisition and expression of fear conditioning.[68-74] Moreover, Patricia and colleagues[75] found that injection of the competitive NMDA receptor antagonist D-AP5 into the dorsal hippocampus impaired the acquisition of context memory without affecting retrieval of fear-related memory. Similarly, antagonizing NMDA receptors with D-AP5 in the basolateral complex prevented acquisition of the context-related memory but had no effect on the expression of fear. Furthermore, infusion of the α-animo-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists CNQX or NBQX into the basolateral complex significantly diminished the expression of fear conditioning.[76,77]

NMDA receptors are composed of two NR1 and two NR2 subunits. The unique electrophysiological and pharmacological properties of the NR2 subunit have made it a focus of interest, including in the study of fear

conditioning. The NR2B subunit blocker Ro25-6981 has been shown to decrease the expression of conditioned fear.[78] Recent work by Corcoran and colleagues[79] found that infusing the NMDA receptor antagonist APV into the retrosplenial cortex impaired retrieval of context fear memory but had no effect on the consolidation or storage of fear memory. Moreover, the retrieval of context fear memory was mediated by the NMDA receptor's NR2A subunit but not by the NR2B subunit. Another study using NR2C knockout mice found that the NMDA NR2C subunit is required for the acquisition of conditioned fear.[80]

Stimulating NMDA receptors can induce activation of an intracellular cascade that ultimately leads to synaptic plasticity. One step of this cascade is activation of protein kinases by NMDA receptors. Studies have shown that protein kinase A, protein kinase C and MAPK inhibitors impair contextual fear memory.[56,81,82] Maren[9] reported that the inhibition of amygdaloid kinase activities by infusions of H7 (a protein kinase inhibitor) selectively inhibits the formation of long-term potentiation and the consolidation of fear memory in the amygdala.[83,84]

2.3.3 GABAergic system

Before SSRIs emerged benzodiazepines were the most widely used anxiolytic drugs. Over the past 30 years many studies have assessed the relationship between GABAergic systems and the neurobiological mechanisms of fear and anxiety. Increased GABAergic transmission has anxiolytic effects[85-87] and disrupts the acquisition or expression of conditioned fear,[88-93] while decreased GABAergic transmission produces anxiogenic-like behavior in animals.[94,95] Fear conditioning acutely decreases extracellular GABA levels in the basolateral complex.[96] Also, genetic studies with presynaptic GABA(B) heteroreceptor knockout mice[97] and GAD65 (a GABA-synthesizing enzyme) knockout mice[98] found that these knockouts resulted in a generalized fear response. Further, transgenic mice with the B3 subunit of the GABA receptor deleted showed impaired acquisition of conditioned freezing.[99]

3. Fear Extinction

Pharmacological and behavior interventions for inhibiting fear and anxiety are important in the management of different types of anxiety disorders. In the fear conditioning paradigm, the inhibition of conditioned fear is called extinction, that is, a reduction in the measured level of fear to a cue previously paired with an aversive event when the cue is repeatedly presented in the absence of the aversive event.[100] Extinction is considered a form of new learning that counteracts the expression of the conditioned fear response, it is not simply forgetting of fear memory.[101] Several reports suggest that similar to the acquisition of fear responses the extinction of fear responses is NMDA receptor-dependent and involves L-type voltage-gated calcium channels (L-VGCCs).[102,103] Fear extinction also produces changes in the intracellular cascade involving kinase and phosphatase activity[104] and protein synthesis.[105,106] For example, Falls and colleagues[103] reported that intra-amygdala infusion of the NMDA receptor antagonist AP5 prevented extinction in a dose-dependent manner. These results are consistent with other experiments in which AP5 and other NMDA antagonists blocked the extinction of contextual fear conditioning, inhibitory avoidance, and eye blink conditioning (a form of fear conditioning performed in humans).[104,107,108] Zimmerman and Maren[109] found that infusion of the NMDA receptor antagonist APV into the basolateral complex or the central amygdaloid nuclei impaired the acquisition of extinction memory. In contrast, the AMPA receptor antagonist NBQX impaired the expression of fear conditioned to an auditory stimulus, suggesting the NMDA and AMPA receptors have different contributions to the expression and extinction of conditioned fear. Taken together, these results suggest that NMDA receptor-mediated transmission plays an important role in the formation and consolidation of extinction.

Numerous reports have indicated that administration of D-cycloserine, a partial agonist of NMDA, either systemically or directly into the amygdala enhances extinction in a dose-dependent manner.[110-113] Furthermore, D-cycloserine reverses fear extinction deficits caused by stress and other factors,[114,115] blocks the extinction-impairing effect of the corticosteroid synthesis inhibitor metyrapone, and enhances the extinction-facilitating effects of the synthetic glucocorticoid dexamethasone.[116] Using electrophysiological approaches Koseki and colleagues[117] found that the spike amplitude in the hippocampal-medial prefrontal cortex pathway is associated with the extinction process for contextual fear conditioning; they also found that the 5-HT1A receptor agonist tandospirone blocked the deficit in fear extinction which occurs in adult rats that had experienced postnatal stress. Importantly, several clinical trials[100,113] find that D-cycloserine facilitates cognitive behavioral therapy in patients with phobia, obsessive-compulsive and panic disorders. These findings suggest that D-cycloserine may be a new pharmacological approach to the treatment of anxiety and fear.

Given the role of the GABAergic system in the acquisition and expression of fear conditioning, it is not surprising that GABAergic interneurons within the amygdala have an important role in the extinction of conditioned fear. Administration of the GABA antagonist picrotoxin after extinction training enhanced prolonged extinction.[118] Administration of diazepam prior to extinction training impaired extinction retention 24h later.[119] Furthermore, an inverse agonist of GABA receptors (which produces the opposite effect of a GABA

agonist) blocked the expression of extinction.[120] A study by Chhatwal and colleagues,[121] found that gephyrin (which regulates GABAergic neurotransmission by clustering GABAA receptors at the synapse) is unregulated after extinction training but down-regulated after fear acquisition, confirming the role of GABAA receptors in both fear acquisition and extinction.

4. Conclusion

The animal model of fear conditioning has made valuable contributions to the understanding of the neurobiological mechanisms underlying the emotional experiences of anxiety and fear. Classical or Pavlovian fear conditioning is the core psychopathology of clinical anxiety disorders, especially post-traumatic stress disorder (PTSD) and specific phobias. Thus investigation of the neurobiological mechanisms involved in the formation, retention and extinction of fear memory is an important step in developing effective treatments for these conditions. A substantial corpus of research using the CFS paradigm has identified the critical regions of the brain and, the synaptic and molecular mechanisms involved in fear conditioning.

Anatomically, the neural circuitry in the amygdala, mainly the basolateral complex, is primarily responsible for associating the conditioned and unconditioned stimuli and the neural circuitry in the hippocampus is primarily responsible for the contextual processing of the stimuli. The synaptic plasticity underlying fear conditioning is NMDA receptor-dependent long-term potentiation in both the amygdala and hippocampus. Antagonizing the amygdaloid and hippocampal NMDA or AMPA receptors reduces the acquisition or expression of fear conditioning. Some specific NMDA receptor subunits may mediate the intracellular cascades of protein kinases within neurons.

The inhibitory effects of GABAergic systems play a critical role in the acquisition and retrieval of fear conditioning. Systemic or local treatments that increase GABAergic transmission produce anxiolytic effects, indicating that local inhibitory circuits in the amygdala contribute to the learning, retention and extinction of fear.

Serotonergic transmission is also related to the neurobiology of fear conditioning. In animals almost all SSRIs show anxiolytic effects, suggesting that 5-HT is involved in the regulation of fear and anxiety. Importantly, the anxiolytic effects of 5-HT-regulating drugs may be mediated by 5-HT1A or 5HT1B receptors within the amygdala or hippocampus that directly or indirectly modulate glutamatergic function via acting on NMDA and AMPA receptors. The 5-HT1A may also regulate the activities of GABAergic interneurons and a number of celluar and molecular events involved in fear conditioning, leading to multiple neurobiological changes in fear learning and memory. Based on these findings, 5-HT1 receptor may be an important target for the development of anxiolytic drugs.

Finally, fear extinction, which requires new learning of fear inhibition, is an important process that is one of the primary determinants of the effectiveness of pharmacological treatments for anxiety and fear. A large body of evidence has revealed that both gluatamatergic and GABAergic systems contribute to fear extinction. Recently, D-cycloserine has been found to facilitate extinction and promote the effects of exposure-based psychotherapy, making it another potential candidate for the treatment of anxiety disorders.

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Conflict of interest

The author reports no conflict of interest related to this manuscript.

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