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REVIEW ARTICLE

Molecular Targets of Anxiety: From Membrane to Nucleus

Long-Jun Wu · Susan S. Kim · Min Zhuo

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Abstract Anxiety is a common human emotional experience that causes decreased quality of life and increased social burden worldwide. However, the treatment options currently available for anxiety are limited as the molecular mechanisms of these complicated emotional disorders are poorly understood. With the development of integrative methods including genetic manipulations, a variety of molecular targets involved in anxiety have been revealed, from membrane receptors, such as 5-HT receptor, $GABA_A$ receptor and GluR5 kainate receptor, and intracellular signaling proteins, such as CaMKIV and AC8, to transcription factors, such as CREB and Egr-1. We propose that all these molecules act together to form a balance between excitatory and inhibitory transmission that is critical for physiological anxiety, and that prolonged disturbance of any of them can promote pathological anxiety-like behavior. Studies on the interactions between these molecules will help elucidate the cellular mechanisms of anxiety, and will provide molecular targets for treating the disorders.

Keywords Anxiety · Fear memory · Amygdala · Synaptic transmission · Excitation/inhibition balance

Introduction

Anxiety is thought to be a way of controlling an animal's response to threatening or potentially threatening stimuli, whereas excessive levels of anxiety, or pathological anxiety, are accompanied by distress and suffering. A common human emotional experience, anxiety can cause people to feel tired, tense and worried, and can strip away the ability to concentrate, sleep, experience pleasure or carry on a peaceful existence. There are at least five forms of anxiety disorders: (1) generalized anxiety disorder, where the patients experience anxiety almost all of the time and cannot identify any particular stimuli triggering it; (2) panic disorder, where the patients experience bouts of panic reaction for no apparent reason; (3) phobias, which are the fear-based, habitual avoidance of anything (person, place, thing, situation, etc.); (4) post-traumatic stress disorder, a condition marked by intrusive, anxiety-provoking memories of trauma; and (5) obsessive-compulsive disorder, in which patients experience incessantly obsessive thoughts and perform repetitive, compulsive acts aimed at alleviating these thoughts and anxiety they produce. Anxiety-related disorders are becoming a leading cause for decreased quality of life worldwide. As many as 25% of adults will, at one point in their lives, suffer from one of the five described forms of anxiety disorders, and the collective economic cost of these disorders is estimated to be over $40 billion per year [1].

The treatment options currently available for anxiety, however, are limited. The most widely prescribed classes of anxiolytic drugs are selective serotonin reuptake inhibitors (SSRIs), which target the serotonergic system, and the benzodiazepines, which modulate the GABAergic system [2]. Animal models of anxiety are useful in determining the mechanism of anxiety disorders. By combining pharmacology and genetics, a variety of molecular targets involved in anxiety have been revealed, from membrane receptors, such as 5-HT receptor and $GABA_A$ receptor, to nuclear transcription factors, such as CREB and Egr-1 [3].

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L.-J. Wu · S. S. Kim · M. Zhuo (✉)
Department of Physiology, University of Toronto, 1 King's College Circle, Toronto, ON, Canada M5S 1A8
e-mail: min.zhuo@utoronto.ca

We believe that all these molecules act together to form a balance between excitatory and inhibitory transmission, which is critical for normal anxiety, while the imbalance caused by disturbing any of these systems can promote pathological anxiety-like behavior.

Behavioral Models of Anxiety

As anxiety is a highly conserved behavior among all species, animal models are widely used to study the molecular mechanism of anxiety disorders. Numerous studies have been conducted on animal models of anxiety to help elucidate the neural mechanisms involved. As animals respond to the potential presence of a threat with characteristic responses and defensive behaviors, investigators use these physiological and behavioral parameters to characterize numerous models of anxiety-like behaviors in animals [1]. The ethologically based animal models of anxiety attempt to create the natural conditions under which such emotional states are elicited, and therefore are thought to minimize possible confounding effects of motivational or perceptual states. However, there still exists individual differences and variable behavioral baseline levels in these models [4]. While there are many animal models of anxiety, only three will be discussed here in detail: the elevated plus maze (EPM), light/dark box, and open field.

One of the best characterized and pharmacologically validated animal models of anxiety is the elevated plus maze [5]. The apparatus has four arms that extend from a central, elevated platform in the shape of a plus. Two opposing arms are enclosed by high walls while the other two opposing arms are unenclosed and have minimal lip. The maze uses conflict between the natural tendency of rodents to explore new environment and fear unfamiliarity, openness, and elevation [6]. The most common measure of anxiety is the percentage of open arm entries or time, and an animal exhibiting a decrease in open arm entries or time would be considered to possess an increased level of anxiety. An example of elevated plus maze test for GluR5 knockout mice is shown in Fig. 1 [7]. The plus maze allows for a rapid screening of anxiety-modulating drugs or mouse genotypes without training or complex schedules, but its behavior patterns may be influenced by variability in test conditions that contribute to discrepancies among results, including age, gender, handling, time of testing, illumination, and method of scoring [4].

Conceptually similar to the EPM, the light/dark box is based on the conflict between a rodent's tendencies to explore a novel environment versus the aversive properties of a brightly lit area [5]. The light/dark test apparatus is divided by a panel into two compartments, one that is brightly illuminated and a slightly smaller one painted black, and the animals are allowed to freely explore both compartments. The most commonly used measures of anxiety are the number of transitions and time spent in the lit chamber, although the latency to exit the dark chamber can also be used as an index of anxiety. An animal exhibiting a decrease in the time spent in the light chamber would be interpreted as possessing an increased level of anxiety [5].

The open field test consists of placing an animal in an unknown chamber with four surrounding walls, and measures a number of behaviors, such as spontaneous exploratory locomotion and the tendency of the animals to prefer the periphery rather than the center of the open field. Although a simple task, there is no standardization between laboratories that use this model: some open fields are square in shape, while others are circular; some are clear and others opaque; some are bright and others totally dark; some have tops and others are open; and presence of objects within the arena, placement of the animal in the open field, the recording period, and items recorded are also variable across the literature. And the pharmacological validation for this task is fairly minimal: as the effects of treatments on exploration are not measured directly so much as the reaction to a stressful event, anxiolytic treatments do not by themselves increase exploration; rather, they decrease the stress-induced inhibition of exploratory behavior [4]. Therefore, although sensitive in most cases to the anxiolytic-like effects of benzodiazepines and 5-HT_{1A} receptor

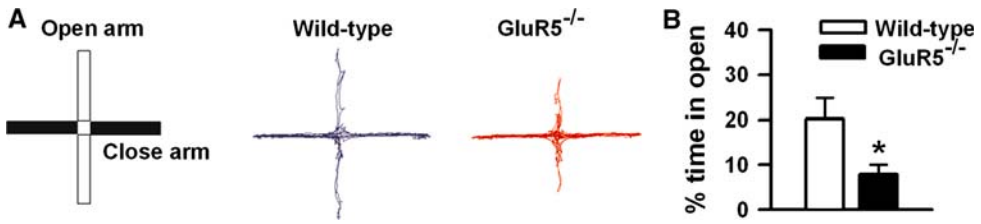

Fig. 1 Elevated plus maze used for testing anxiety-like behavior in mice. (a) From left to right: Diagram of the EPM (left), filled boxes = closed arms, open boxes = open arms; representative traces showing the movement of wild-type (middle) and GluR5 knockout ($GluR5^{-/-}$) mice (right) in the elevated plus maze for 5 min. (b) Pooled data indicated that $GluR5^{-/-}$ mice spent significantly less time in the open arms of the elevated plus maze compared to wild-type mice, suggesting increased anxiety-like behavior in the knockout mice

agonists, compounds that are clinically used as treatments for anxiety disorders, such as panic attacks, generalized anxiety disorder or obsessive-compulsive disorder are ineffectual in this task, suggesting that this paradigm may not model all features of anxiety disorders [8].

Neural Circuitry of Anxiety: Central Role of the Amygdala

Since anxiety is regarded as an innate fear, the structural basis of anxiety resides in the neural circuitry related to fear response. The amygdala is a key structure for processing neuronal inputs from other parts of the brain, initiating output signals to responding nuclei, and generating various physiological responses, including behavioral, autonomic, and hormonal responses related to anxiety [9]. In both humans and animals, electrical stimulation of the amygdala elicits anxiety, whereas lesion of the amygdala impairs the perception of fear [10]. In addition to the amygdala, various studies using lesion, microinjection, electrophysiology or imaging techniques have implicated other brain regions in anxiety, including cortex, hippocampus, hypothalamus, and brainstem [1].

Anxiety is thought to share the neural circuitry of conditioned fear (Fig. 2). Anatomically, information from sensory modalities reaches the amygdala via projections from the thalamus, cortex or hippocampus. There are intra-nucleus interactions for the transmission and integration of the sensory input in the amygdala. Essentially, the information flows from the lateral amygdala to the basolateral amygdala, before finally reaching the central amygdala. Then the efferents from central amygdala go to the periaqueductal gray, brainstem and hypothalamus, which initiate fear-related behavioral, autonomic and hormonal responses [11, 12]. Figure 2 shows this simplified information flow, from transmitting threat stimulus to initiating anxiety/fear behaviors. The normal function of this circuit is critical for physiological anxiety, while the dysfunction of this circuit will lead to pathological anxiety. We have to keep in mind that there are complex interactions between these brain regions underlying different aspects of anxiety, such as memory, aversion, and motivation. Moreover, we propose that the local interneurons will also affect the information flow between different brain regions (Fig. 3).

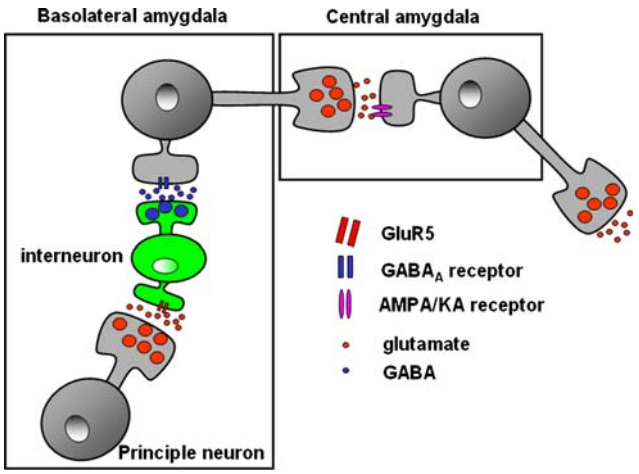

Fig. 3 Proposed model for the anxiolytic function of GluR5 kainate receptor in the basolateral amygdala. In the basolateral amygdala, GluR5 is mainly expressed in somatodendritic regions of interneurons. Activation of GluR5 initiates action potential firing that increases GABA release. The released GABA produces feed-forward inhibition on pyramidal neurons and the subsequent output to the central amygdala. Therefore, activation of GluR5 in the basolateral amygdala reduces the information flow transmitted to the central amygdala, which may explain the increased anxiety-like behaviors in GluR5$^{-/-}$ mice

Molecular Targets of Anxiety: Membrane and Intracellular Signaling Proteins

Pharmacological and electrophysiological methods have allowed researchers to make testable predictions about the molecules involved in anxiety. However, pharmacological interventions have often been questioned for its selectivity. With the development of genetic manipulations, i.e., genetic knockout and transgenic mice, the number of molecular targets for anxiety have been growing tremendously [3]. The combination of pharmacological and genetic manipulations

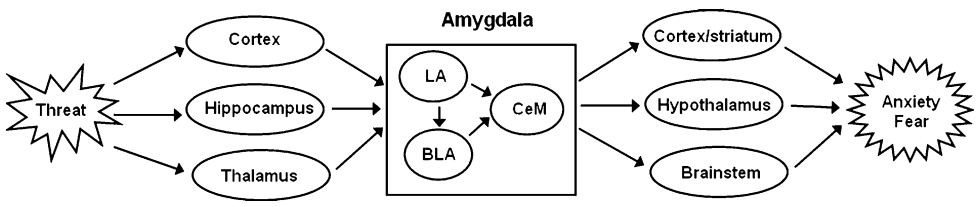

Fig. 2 A simplified diagram of neural circuitry for anxiety. Threatening stimuli are detected by different brain areas, such as thalamus, hippocampus and cortex. The information is then transmitted to the amygdala, where it is relayed and integrated in different nuclei. The output is then transmitted from the amygdala to brain areas, such as cortex/striatum, hypothalamus, and brainstem, initiating fear/anxiety responses. LA, lateral amygdala; BLA, basolateral amygdala; CeM, central amygdala

provide more convincing evidence that GABAergic, glutamatergic, serotonergic, noradrenergic, and corticotropin-releasing hormone (CRH) systems are linked to anxiety behaviors [5, 13]. We propose that pathological anxiety is due to the imbalance of excitation/inhibition in neural circuitry underlying physiological anxiety. Therefore, the dysfunction in neurotransmitter systems will lead to abnormal excitability of the neuronal network, thereby causing aberrant anxiety responses.

Since there are a large number of molecules involved in anxiety, we will focus mainly on the recent progresses made in gene mutation-associated anxiety behaviors. We have separated these targets into three categories based on their cellular location: the membrane proteins, cytoplasmic proteins and nucleic proteins. Membrane proteins, such as ionotropic (i.e., $GABA_A$ receptor, GluR5 kainate receptor) and metabotropic receptors (i.e., 5-HT_{1A} receptor, CRH receptor 1) have been intensively studied and reviewed in anxiety disorders [3, 13], while intracellular signaling proteins including enzymes (i.e., glutamate decarboxylase 65 and monoamine oxidase A), signaling proteins (i.e., CaMKIV, AC8), and activity-dependent immediate early genes (e.g., Egr-1, CREB) are emerging, providing new insights into the molecular mechanisms of anxiety. In each of these categories, we will give examples of how these molecules are integrated into the neural circuits and their effects on anxiety-like behaviors.

Membrane Proteins: Ionotropic Receptors

$GABA_A$ Receptor

A few of the commonly used anxiolytic drugs, such as benzodiazepines and anticonvulsants, are $GABA_A$ receptor modulators. Genetic alterations at the level of individual $GABA_A$ subunits provide powerful information for its subunit-dependent role in anxiety. For example, α1-subunit mutant mice are insensitive to the sedative and anticonvulsant effects of diazepam, but remain responsive to its anxiolytic effects [14], while δ-subunit knockout mice are insensitive to the anxiolytic effects of neuroactive steroids [15]. Spontaneous anxiety-like behavior is also found in γ2-subunit knockout mice [16]. These results suggest the positive roles of δ- and γ2-subunits, but not α1-subunits, in mediating anxiety. The role of $GABA_A$ receptor in anxiety is clear, as activation of $GABA_A$ receptor reduces the neuronal and network excitability. However, the reason for the distinct roles of different subunits of $GABA_A$ receptor in anxiety is still debatable. One possible explanation is that the different subunits of $GABA_A$ receptor may mediate phasic or tonic components of GABA current, which have different kinetics in current, ligand affinities, etc. [17].

GluR5 Kainate Receptors

Glutamate receptors have a rich diversity of localizations and functions within the central nervous system. Therefore, the challenge of using glutamate receptors in anxiolytic drugs is determining the appropriate targets and achieving specificity. Recently we have found that GluR5-containing kainate receptors could exert its selective role on GABA release in the basolateral amygdala, and thus serve as a possible target for controlling anxiety-like behaviors [7]. Knockout of GluR5 or selective blockade of GluR5 in the basolateral amygdala increased anxiety-like behavior while selective activation of GluR5 decreased it. In vitro slice recording showed that GluR5 is selectively expressed in interneurons, and its activation could largely depolarize those interneurons and increase synaptic GABA release as well as GABA tonic currents. More importantly, the GluR5 activation in the basolateral amygdala reduced the output to the central amygdala, which may explain the increased anxiety phenotype in the GluR5 knockout mice [7]. We also found similar roles of GluR5 in the anterior cingulate cortex (ACC) [18], suggesting a common role of GluR5 in anxiety-related neural circuitry. Interestingly, we found that GluR6 knockout mice showed reduced fear memory but not anxiety, which may be due to its role in the induction of long-term potentiation (LTP) in the lateral amygdala [19]. Therefore, GluR5 but not GluR6 is selectively engaged in anxiety-like behaviors in the amygdala.

Membrane Proteins: Metabotropic Receptors

5-HT_{1A} Receptor

Serotonergic system is intensively implicated in anxiety. In particular, SSRIs are the first-line compounds for clinical treatment of anxiety-related disorders. Among the various 5-HT receptor subtypes, the 5-HT_{1A} receptor is well studied for its role in anxiety. In 1998, three independent groups found that knockout of 5-HT_{1A} receptor led to an anxiogenic phenotype in the open field, EPM, zero maze, and novelty-suppressed feeding task [20–22]. Using a tissue-specific, conditional rescue strategy, Gross et al. further showed that expression of 5-HT_{1A} receptor, primarily in the hippocampus and cortex, is sufficient to rescue the behavioral phenotype of the 5-HT_{1A} receptor knockout mice. Furthermore, they showed that the receptor expression during the early postnatal period is necessary for the rescue [23]. The mechanism resides in membrane hyperpolarization and decreased neuronal excitability by activation of 5-HT_{1A} receptors, which is coupled to potassium channels [24].

CRH Receptor 1

Corticotropin-releasing hormone (CRH) has been shown to act as a neurotransmitter or neuromodulator in the brain, and is known to be involved in anxiety-like behaviors. The mutant mice with deletion of CRH receptor 1 displayed reduced anxiety in several tests, such as the EPM and the light/dark box [25, 26]. Forebrain and limbic system-specific CRH receptor 1 knockout mice exhibited less anxiety similar to the conventional knockout mice [27]. These results suggest that CRH receptor 1, particularly the limbic-specific receptor, is important in mediating anxiety-like behaviors. It has been reported that activation of CRH receptor 1 could enhance GABAergic neurotransmission in neurons from the central amygdala [28], which might explain the role of CRH in anxiety.

Cytoplasmic Proteins: Enzymes

GAD65

Extracellular GABA concentration is important in maintaining inhibitory tone, which plays a role in anxiety. For example, neuroimaging studies have demonstrated a clear reduction of GABA levels in the occipital cortex of patients with panic disorders [29]. Mice deficient in the GABA-synthetic enzyme, glutamate decarboxylase (GAD) 65, have reduced levels of the neurotransmitter and show increased anxiety-like behavior in the elevated zero maze and open field [30].

MAO-A

Monoamine oxidase A (MAO-A) is critical for the metabolism of dopamine, norepinephrine, and 5-HT. Mutant mice lacking the MAO-A gene have been reported to have an elevated brain content of norepinephrine and 5-HT, and exhibit reduced anxiety-like behavior in the open field [31].

Cytoplasmic Proteins: Signaling Proteins

CaMKIV

The Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) is a serine-threonine kinase that is activated by elevated intracellular Ca^{2+} during neuronal activity. The downstream proteins for CaMKIV include CREB and CRE modulator [32]. CaMKIV is known to be important for gene expression, synaptic plasticity, and learning and memory. For example, deletion of CaMKIV affects long-term potentiation in the lateral amygdala and fear memory [33]. Recently, we have found that CaMKIV knockout mice exhibited decreased anxiety-like behavior in both the elevated plus maze and light/dark box, suggesting a key role for CaMKIV in anxiety [34]. Microarray analysis has found downregulation of some anxiety-related gene in CaMKIV knockout mice, such as oxytocin and vasopressin [35, 36], which may explain its anxiety phenotype.

AC8

Calcium-stimulated adenylyl cyclase type VIII (AC8) is one of AC isoforms important for the production of cAMP and activity-dependent gene regulation. Unlike AC1, AC8 is highly expressed in the thalamus, habenula, and hypothalamus, brain regions involved in the neuroendocrine and behavioral responses to stress [37]. Although naïve AC8 knockout mice exhibit normal anxiety, they show less stress-induced anxiety [38]. The actions of AC8 in stress-induced anxiety may be due to its role in stress-induced CREB activation, gene expression, as well as long-term depression.

Activity-Dependent Immediate Early Genes and Transcription Factors

CREB

cAMP-responsive element-binding protein (CREB) is well known for its role in activity-dependent gene regulation and is involved in many neuronal processes, including synaptic plasticity and memory. Recently, it was found that CREB is also engaged in emotional behaviors, such as anxiety. CREB knockout mice exhibited increase in anxiety-like behaviors in several paradigms including the elevated plus maze, light/dark box and open field [39]. The mechanism by which CREB is involved in anxiety is not known, but it has been proposed that CREB could regulate the neuropeptide Y system (NPY) expression, and decreased concentrations of NPY are implicated in anxiety-like behaviors [40].

Egr-1

The zinc finger transcription factor Egr-1 is critical for coupling extracellular signals to changes in cellular gene expression [41]. Using Egr-1 knockout mice, we have found that it is selectively required for synaptic potentiation in the amygdala, late auditory fear memory and anxiety [42]. Egr-1 knockout mice showed reduced anxiety behavior compared with wild-type mice in the elevated plus maze [42]. The exact mechanism by which Egr-1 mediates anxiety is currently unknown, but it is likely some

downstream gene expression regulated by Egr-1, such as synapsin I and II, would play a role.

Cellular Models of Anxiety: Balance of Excitation and Inhibition

As we have mentioned above, various neurotransmitter or neuromodulator systems, such as GABAergic, glutamatergic, serotonergic, noradrenergic, and corticotrophin-releasing hormone systems, as well as intracellular signaling proteins, were shown to be involved in anxiety-like behaviors. Genetic mutation or pharmacological manipulations of one or combination of them could lead to abnormal levels of anxiety. These findings indicate the complexity of the cellular and molecular mechanisms underlying emotional behaviors, such as anxiety. A balance between excitatory and inhibitory transmission is critical for normal brain function. Hyperexcitation due to enhanced excitatory transmission or reduced inhibitory transmission can promote anxiety-like behavior [11]. Therefore, we propose a cellular model based on the concept that pathological anxiety is due to the imbalance of excitation and inhibition in neural circuits, with the amygdala playing a central role.

We will focus on the roles of GluR5 (Fig. 3). Previous studies have provided clear evidence that the basolateral amygdala is an important input station for the integration of thalamic and cortical sensory afferents, while the central amygdala is a major output station in regulating fear and anxiety responses [12]. Moreover, anatomical and electrophysiological studies have demonstrated the presence of excitatory projections from the basolateral amygdala to the central amygdala, forming a major intra-amygdaloid circuit [43, 44]. We have shown that activation of GluR5 in the basolateral amygdala has an inhibitory effect on the basolateral amygdala network excitability, which would then affect synaptic transmission from the basolateral amygdala to the central amygdala. Additionally, knockout or antagonism of GluR5 increased the synaptic transmission from basolateral amygdala to central amygdala. Consistently, we have observed increased anxiety in GluR5 knockout mice and decreased anxiety by activating GluR5 [7]. Therefore, we conclude that manipulation of GluR5 changes the balance of excitation and inhibition in the basolateral amygdala, thereby affecting the amygdalar output and anxiety-like behaviors.

If all molecules that play a role in anxiety are integrated into the same picture, we believe that every neurotransmitter and neuromodulator system or intracellular signaling protein could contribute to the balance of excitation and inhibition, thereby exerting their effects on anxiety responses. The location of these targets could be in any nuclei in the neural circuits underlying anxiety.

Conclusion and Future Directions

In this review, we have described behavioral models, neural circuitry, and molecular targets of anxiety, with a listing of the proteins involved in anxiety from membrane to nucleus. Finally, we have proposed a cellular model for the generation of anxiety, where all the molecular targets converge to the same point: the balance between excitation and inhibition of the network.

Understanding the role of various molecular targets in anxiety disorders will help to address the etiology of anxiety and lead to development of novel treatments. Genetic manipulations provide a powerful tool in accomplishing this goal. However, the data obtained with these manipulations may not take into account compensatory mechanisms, which occur during development, or interactions between different brain regions. Techniques, such as RNA interference, which could robustly and selectively induce suppression of specific genes of interest, could be used to minimize compensation and offer spatial and temporal selectivity. Certainly the integration of techniques including genetics, electrophysiology, biochemistry and neuroimaging will improve potential findings for the possible molecular targets and validate the conclusions.

Although a tremendous range of potential targets for the development of new anxiolytic compounds has been proposed, there are still only a few drugs that are clinically used to treat anxiety in patients. The translational work from preclinical experiments to clinical trials still has a long way to go. Changing the traditional ideas from membrane targets to intracellular signaling proteins might give new directions for the design of novel anxiolytic compounds with fewer side effects.

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