

Review

# Different patterns of freezing behavior organized in the periaqueductal gray of rats: Association with different types of anxiety

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## Abstract

Freezing defined as the complete absence of body movements is a normal response of animals to unavoidable fear stimuli. The present review presents a series of evidence relating different defensive patterns with specific anxiety disorders. There are at least four different kinds of freezing with specific neural substrates. The immobility induced by stimulation of the ventral column of the periaqueductal gray (vPAG) has been considered a quiescence characteristic of the recovery component of defense–recuperative processes. There is an isomorphism between freezing response to contextual stimuli paired with electrical shocks and generalized anxiety disorder. Besides, two types of freezing emerge with the electrical stimulation of the dorsal aspects of the periaqueductal gray (dPAG): the dPAG-evoked freezing and the dPAG post-stimulation freezing. Evidence is presented in support of the hypothesis that whereas dPAG-evoked freezing would serve as a model of panic attacks, the dPAG post-stimulation freezing appears to be a model of panic disorder. It is also proposed that conditioned freezing plus dPAG electrical stimulation might also mimic panic disorder with agoraphobia. A model of serotonergic modulation through on- and off-cells of the defense reaction generated in the dPAG is also presented. The understanding of how the periaqueductal gray generates and elaborates different types of freezing is of relevance for our better knowledge of distinct types of anxiety such as panic disorder or generalized anxiety disorder.

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**Keywords:** Animal models of anxiety; Freezing behavior; Generalized anxiety disorder; Panic attacks; Panic disorder without agoraphobia; Panic disorder with agoraphobia

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## 1. Introduction

Anxiety disorders are among the most prevalent mental health problems across the individual life span. Animal modeling has been crucial in dissecting pathophysiological mechanisms and designing more effective therapies. A difficulty in modeling psychiatric disorders has been limited information about their origin and underlying neural mechanisms. Although anxiety research is no different from any other psychiatric disease this is likely in better position to be modeled than any other psychiatric disease, given that the circuitry is well-understood and maintained in many ways between humans and rodents [48]. Based on these animal models, experimental studies suggest that different defensive patterns, which involve well-defined neural circuitries, might be associated with specific anxiety disorders [18,40,41]. Thus, it has been possible to find correlation between these disorders and defensive responses that animals present when facing with dangerous situations. The purpose of the present article is to review some of these studies, which point out that the neural systems underlying distinct patterns of defensive freezing behavior might be associated with different anxiety disorders.

The first section of this review makes an appraisal of how anxiety disorders are currently classified according to nosological categories. The second and third sections discuss how different animal defensive behaviors and their respective neural circuitries might be related to generalized anxiety disorder (GAD) and panic disorder (PD). The fourth section describes a series of results showing that there are at least four types of freezing behavior associated with different patterns of defensive behaviors. A hypothesis is also discussed that proposes that the dorsal periaqueductal gray (dPAG)-evoked freezing is a model of panic attacks, whereas the dPAG post-stimulation freezing is a model of PD. It is also suggested that freezing behavior triggered by contextual cues previously associated with dPAG electrical stimulation might be a model of PD with agoraphobia. The fifth section presents pharmacological results obtained with animal models of anxiety designed to examine the chemical mediation of the several types of freezing associated with the PAG. The last section summarizes the reviewed evidence and states the conclusions.

## 2. Historical background

To make an integrative appraisal of the neural circuits proposed to underlie the different types of anxiety, we start with important changes that took place in the classification of anxiety disorders during the 1970s. During that time, most psychiatrists viewed anxiety as a single construct that ranged in intensity from normal to pathological or neurotic levels. Accordingly, anxiolytic drugs were the main prescription to treat this single disorder. A major shift to this view occurred in the beginning of 1980, with the publication of the 3rd edition of the American Psychiatric Classification (DSM-III) [1]. Some years later, the revised version of this classification established PD as an independent nosological category [2]. This new classification delineated distinct nosological entities, namely, GAD, PD, agoraphobia, simple phobias, social phobia, post-traumatic stress

disorder, and obsessive–compulsive disorder. In general, the same proposal remained in the DSM-IV classification, being also adopted by the ICD-10 classification of the World Health Organization of 1992 [85].

Among the diverse types of anxiety, GAD and PD, with and without agoraphobia, are the nosological entities focused in this review. GAD is diagnosed when a patient worries excessively about a variety of everyday problems. The worry construct has received considerable attention since the diagnostic of GAD shifted from a residual category in the DSM-III [1] to an independent anxiety disorder type in the 4th edition of the DSM [3]. According to the DSM-IV, worry is generally associated with several symptoms such as muscle tension, feeling tired and restless, concentration difficulties and irritability. These worries are generally associated with impairments in academic, social, or personal functioning and related to multiple domains or activities. In order to be considered a pathological feature of GAD, worry must occur (more days than not) for a period of at least 6 months.

Panic attacks are sudden surges of intense fear or terror, desire of fleeing and feeling of imminent death or losing control. These subjective symptoms are accompanied by major neurovegetative changes, such as palpitation, hypertension, difficulty in deep breathing, sweating, urge to void the bladder and increased peristalsis. Panic disorder, with or without agoraphobia, is a psychiatric illness that can have a chronic course and be associated with significant morbidity. PD is characterized by recurrent panic attacks, either unexpected or associated with particular situations, and persistent concern about having another attack or worry about the implications and consequences of the panic attacks. This leads to worry about the next attack or anticipatory anxiety, and avoidance of places where a panic attack would be embarrassing [40,41].

Panic disorder, with agoraphobia, may lead to the loss or disruption of interpersonal relationships, especially as individuals struggle with the impairment or loss of their social role and the issue of responsibility for symptoms. Patients with PD frequently fear that panic attacks represent catastrophic medical events despite the evaluation indicating the absence of a medical problem. Patients also fear that the attack is an indicator of a life-threatening illness. In addition, they may live in a nearly constant state of apprehension and may be severely limited by phobic avoidance. Patients with PD with agoraphobia experience anxiety and avoidance of places or situations where escape or help may be unavailable if they have panic symptoms. Typical situations eliciting agoraphobia include traveling on buses, subways, or other public transportation and being on bridges, in tunnels, or far from home. Many patients who develop agoraphobia find that situational attacks become more common than unexpected attacks. Some patients might experience severe attacks so that they might take extreme actions such as quitting a job to avoid the possibility a new attack. Others may become so anxious that they eventually avoid most activities outside their homes [3].

Panic attacks are among the most prominent symptoms in PD, but they might occur in other anxiety disorders. Panic attacks are discrete periods of intense fear or discomfort, accompanied by at least 4 of the 13 somatic or cognitive symptoms defined

by DSM-IV [4]. An attack has an abrupt onset and reaches a peak usually within 10 min. It is often accompanied by a sense of imminent danger and an urge to escape. Panic attacks vary in their frequency and intensity. Other types of panic attacks include those that occur in particular emotional contexts, those involving limited symptoms, and nocturnal attacks. Patients with posttraumatic stress disorder, obsessive–compulsive disorder, generalized anxiety disorder, and specific and social phobias also sometimes report occasional panic attacks. Identification of triggers for panic attacks is important, since a patient can be misdiagnosed with panic disorder. Whereas panic attacks refer to an acute crisis PD has the “worries” about new crises as the subjective component [1].

Although patients with PD share common features of the illness, there are important interindividual differences. The frequency of panic attacks varies widely among patients, and the constellation of symptoms for each attack also differs. Some patients complain, for example, of attacks that primarily involve cardiovascular symptoms, such as palpitations, chest pain, and paresthesia, while others are more overwhelmed by cognitive symptoms, such as depersonalization and the fear of “losing one’s mind”. The amount of anticipatory anxiety and the degree of phobic avoidance also vary from patient to patient. Many patients with PD are not highly avoidant. At the opposite extreme, there are patients who will not leave the house without a trusted companion. Thus, an appreciable degree of variability is a common feature in PD [45–47].

It is important to bear in mind the difficulties basic researchers are faced with when attempting to use the current classifications of mental disorders as a foundation for developing viable animal models. DSM and other available diagnostic and classification systems provide a partially validated mechanism whereby physicians can provide reliable diagnoses, communicate amongst themselves, and report their findings to insurance providers. However, these classification approaches are not primarily based upon etiology, neurobiology, epidemiology, genetics, or response to medications, but rather on gross behaviors that have imprecise similarity and/or correlation with each other within and between individuals. Disease heterogeneity implicit in the current classification schema, and the imprecise quantification of the behaviors being described, makes it difficult to even partially deconstruct such “diseases” within model organisms (see [37], for extensive discussion).

### 3. Levels of defensive reaction

As discussed above, anxiety disorders constitute a heterogeneous group of related nosological categories. Different animal models of anxiety have been employed as useful tools for investigating and understanding the underlying mechanisms for different anxiety disorders. An important step in this field of defensive behavior research was undertaken by Blanchard et al. [7], who carried out systematic studies on the defensive strategies adopted by wild rats facing different types of predatory threat. The obtained results led to the concept of three levels of danger – namely potential (uncertain), distal and proximal threat – each evoking a different type of defense reaction. For instance,

rats perform cautious exploration aimed at risk-assessment when danger is uncertain, like in novel environments. When the predator is perceived at distance, tense and attentive immobility (freezing) ensues. Finally, when the predator is near or in actual contact with the rat, the animal flees whenever possible or otherwise threatens back or even attacks the predator defensively [6]. Comparative studies led to the conclusion that homologous types of defense strategies can be found in other animals, including non-mammalian species [8].

Bolles and Fanselow [10,11] also developed a similar model of defensive behavior employing the traditional context fear conditioning paradigm. For example, when a rat is placed in a novel context and some minutes later an inescapable electrical footshock is presented, the animal reacts with an extremely vigorous activity including escape attempts, jumping and vocalization responses. At the end of the shock, this pattern of activity burst gradually gives way to a period of freezing [9]. According to this model, these two different defensive responses are mediated by two independent motivational systems—one related to fear and the other related to pain. The fear-motivational system is activated by distal stimulus that might impose some danger to the animal. A pattern of inhibitory response, freezing, is the main defensive behavior triggered by this system. On the other hand, the pain-motivational system is activated by proximal nociceptive stimuli and serves to protect the animal from body injury through active forms of behavior, such as jumping and running. Additionally, this painful stimulus acts as an unconditional stimulus that produces an immediate context fear conditioning. Therefore, the freezing response that ensues when the shock ends is related to the fear-motivational system which is activated by the contextual cues paired with the shock. It is interesting to note that Blanchard’s first and second levels of defense are analogous to Bolles and Fanselow’s fear-motivational system whereas the Blanchard’s third level of defense appears to be related to Bolles and Fanselow’s pain-motivational system. The theoretical construct backing these two fear models is the start point to new anxiety/panic models based on different kinds of freezing with specific neural substrates as discussed later in this review.

### 4. The neural substrates of defensive behaviors

Although the present knowledge about the neural substrate of these defense strategies is still incomplete, there have been attempts to relate each level of defense with a set of brain structures. In general, the septo-hippocampal system and the defense brain system have been proposed to organize conditioned and unconditioned fear, respectively, in the brain. Conciliating these two lines of evidence, Gray and McNaughton [42] have argued that the septo-hippocampal system would contribute with the cognitive component (worry), whereas the amygdala, mainly involved with the defense brain system, would impart the affective tone to distal threatening stimuli. The septo-hippocampal system and the amygdala would be the key structures for risk-assessment and defensive freezing behavior in response to innate potential threat and conditioned stimuli. Thus, as can be seen in Fig. 1 even though conditioned and innate fear stimuli acti-

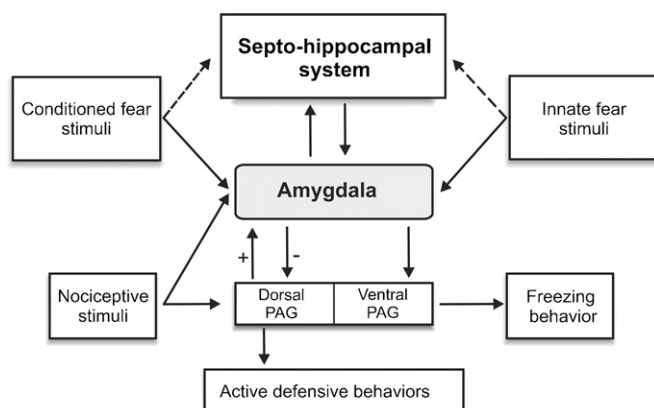


Fig. 1. Activation of distinct neural substrates in function of the nature of the threatening stimuli. Conditioned (potential or distal) danger stimuli elicit conditioned freezing through activation of the behavioral inhibition system (septo-hippocampal system) whereas innate danger stimuli (proximal or nociceptive) elicit active forms of defensive behavior through activation of the amygdala–dPAG axis. Activation of the circuit dPAG–amygdala by nociceptive stimuli may also trigger inhibitory mechanisms through amygdala–dPAG projections. This may underlies the so-called fear-induced analgesia or the proposal that anxiety inhibits panic.

vate the septo-hippocampal system (dotted lines) and amygdala (solid lines) these structures might be related to different defensive behaviors.

The septo-hippocampal system would provide the cognitive component of anxiety while the affective component would be integrated in the amygdala [42]. Projections from the amygdala to the ventral portion of the PAG (vPAG) would be associated with the occurrence of defensive freezing. Indeed, Fanselow [31–33] suggested that the amygdala would synthesize stimuli input from the environment and then signal to the vPAG the degree of threat that they represent to the organism. This longitudinal and hierarchically organized neural system would be in charge of selecting, organizing and executing the appropriate behavioral and neurovegetative defensive reactions. It is possible that the malfunctioning of this defensive system might be related to pathological forms of anxiety present in GAD.

Besides this neural circuitry associated with inhibitory defensive behaviors, there is also a different system responsible for a completely opposite pattern of defensive behavior. It involves extremely vigorous forms of active behavior, such as flight-or-fight reactions to proximal danger stimuli, generally associated with nociception. The dPAG seems to be the main structure responsible for organizing these active defensive behaviors, since it receives major projections from neuronal pathways associated with pain processing. Projections from the amygdala to the dPAG would be implicated in this defensive reaction so as conditioned danger stimuli previously associated with nociceptive stimulation could activate the amygdala and thus trigger a defensive freezing response from the dPAG.

It is important to point out that the amygdala and the dPAG might work in an opposite manner. Accordingly, dPAG activation through nociceptive stimulation might in turn active the amygdala to produce fear conditioning, whereas amygdala activation through innate or conditioned danger stimuli might in

turn dump active defensive behaviors associated with the dPAG. Indeed, it has been reported that context fear conditioning, which involves the activation of the amygdala, can trigger an analgesic reaction that can inhibit withdrawal reflex to painful stimuli, such as tail-flick to radiant heat, or more complex and elaborated nociceptive responses elicited in the formalin test [32]. Moreover, it has been shown that context fear conditioning can inhibit vigorous running and jumping triggered by footshock [32] as well as shock-induced defensive fight [9]. Interestingly, context fear conditioning can also inhibit the occurrence of defensive escape responses evoked by dPAG electrical stimulation [55].

From what has been discussed above, it can be assumed that the ventral and dorsal portions of the PAG play different roles in defensive reaction. Whereas the amygdala–vPAG axis appears to be involved in the occurrence of conditioned freezing behavior, the neural substrates of fear in the dPAG appears more associated with active forms of defensive behavior. Delgado et al. [29] showed that laboratory animals readily learn to operate a device that switches off an electrical current delivered in the dPAG. We also confirmed that the electrical stimulation of the dPAG produces strong aversive reactions, which were attenuated by local injections of morphine [15,16]. It is now clear that electrical stimulation of the dPAG in laboratory animals induces cardiovascular changes including an increase in heart rate and blood pressure, hyperventilation, and freezing behavior alternating with running and jumping, at higher stimulation intensities [16]. These defense strategies are expressed in natural conditions when a predator is very close to or in direct contact with the prey [8]. Accordingly, in terms of face validity and pharmacological predictability these manifestations of unconditioned fear have been proposed as an animal model of panic attacks [27,77]. In consequence, malfunctioning of the dPAG, the core structure responsible for these defensive reactions, might be related to panic attack. Indeed, the dPAG is the most likely option for organizing escape behavior evoked by proximal threat. Drugs acutely reducing (alprazolam, clonazepam) or precipitating (yohimbine, caffeine) panic attacks in patients were also found to acutely and dose-dependently reduce or enhance, respectively, the aversion induced by dPAG stimulation in rats [45]. Besides, as it will be described later in this review, a role for serotonin in aversion has been suggested by studies investigating the effects of serotonergic drugs on the aversive threshold of electrical stimulation of the dPAG [12,20,45–47,77]. Using this model it has been found that the antipanic agents, fluoxetine and zimelidine, reduced the defensive response in rats subjected to the dPAG stimulation procedure [12,46]. These compounds attenuate the behavioral effects of dPAG stimulation, probably because they enhance the inhibitory function of neuronally released 5-HT, which has been reported to decrease the frequency of panic attacks [38,80]. Thus, the association between dPAG stimulation and panic has received considerable support from pharmacological studies. The frequent link made between dPAG stimulation-elicited flight and panic has not been extended to the freezing induced by this same stimulation because few laboratories have also looked at measuring the defense reaction in its successive behavioral steps; alertness, freezing and escape [12,20,40,77]. However, studies using this approach have also shown that compounds that acting



by increasing the 5-HT transmission in the dPAG also reduce the freezing evoked by its electrical stimulation [12,67].

In agreement with this view, fight-or-flight reactions are also induced by cutaneous nociceptive stimuli or by suffocation [27], and a low suffocation threshold has been implicated in the genesis of panic attacks that occur in PD patients [51]. In this regard, electrical stimulation of the dPAG has been reported to induce panic-like symptoms in neurosurgical patients. These were palpitation, blushing of face and neck and respiratory arrest or hyperventilation, feelings of terror or impending death, and desire to flee [63]. Such evidence, among others, led to the suggestion that the dPAG may be involved in PD [27,39,54]. However, the notion that a given structure or particular neurochemical mechanisms could be associated with different types of anxiety was achieved only after important changes in both clinical and pre-clinical theoretical frameworks had taken place.

A caveat in the study of the neurobiology of defensive behavior was that the attempts to correlate the above levels of predatory defense to anxiety-related disorders have somehow neglected the freezing behavior induced by the electrical stimulation of the dPAG in diverse experimental circumstances. Moreover, the dPAG has been considered as the main output center for the defensive behavior despite earlier studies that endorse the possibility of processing of information of aversive nature at this midbrain level ([19,20], for reviews). In this review, similar attention is given to the sensory as well as the expression of defensive behaviors triggered by dPAG stimulation and, in particular, to the aversive states underlying certain types of freezing behavior.

## 5. Neural substrates of freezing

Freezing is defined as the complete absence of body movements, except those necessary for respiration [9]. Many reports show that freezing is the most common defense response in rats exposed to unavoidable fear stimuli [58]. In this section we describe four categories of freezing. The conditioned freezing and tonic immobility are two types of defensive behavior associated with the neural substrates of fear of the vPAG. The dPAG-evoked freezing – lowest intensity of the dPAG electrical stimulation producing freezing – and the dPAG post-stimulation freezing – duration of freezing after the interruption of the electrical stimulation of the dPAG – have been considered as two new types of freezing with distinct biological functions [67,72,73].

The freezing elicited by stimulation of the vPAG can be inhibited by local injections of midazolam into this region. To characterize whether the anti-freezing effect of midazolam injected into the vPAG was due to its known anxiolytic action, rats given midazolam into the vPAG were submitted to the elevated plus-maze test (EPM). The main effect of midazolam was an increase in the exploratory activity in the closed arms of the maze without any significant change in entries into or time spent in the open arms [26]. If the reduction in freezing caused by midazolam was due to the anxiolytic properties of this drug, then it would also reflect in a reduction of the avoidance of the open arms of the EPM. However, this was not the case. The increase of the general motor activity caused by injections of

midazolam into the vPAG could not be attributed to diffusion of the drug to the dorsal PAG either. The selectivity of this effect is attested by the fact that clear anxiolytic effects were obtained when midazolam was similarly injected into the dPAG of rats submitted to the EPM test [61]. Although further studies are still needed, two possibilities can be raised to explain these findings. First, vPAG stimulation prevents an active response by imposing quiescence, which is reduced by midazolam. The immobility induced by stimulation of the vPAG has been considered a kind of quiescence characteristic of the recovery component of the defense–recuperative process following injuries [49]. The activity evoked by injections of midazolam into the vPAG could simply be the result of disinhibition of this motor system with the release of the exploratory behavior and the loss of the imposed quiescence [60,84].

Another view considers that the vPAG merely would mediate the motor aspects of immobility which is in turn being modulated by the amygdala [21,65,66]. Thus, inactivation of the vPAG, as occurred with local injections of midazolam, would increase animal's general activity without altering fear. In fact, this drug increased entries in the EPM, regardless of the type of arms. Thus, the vPAG-linked immobility would not be triggered by unconditional stimuli such as the height and open spaces of the EPM, so as they bypass the vPAG in the production of the characteristic open arms avoidance [18,26]. This second reasoning implies that conditioned fear is clearly modulated by the amygdala–vPAG connections. Indeed, the reversible inactivation of these structures produced by local injections of muscimol or lidocaine reduces the conditioned contextual freezing [21,52,56].

Analyses of animal distress behaviors suggest that tonic immobility is also an important defensive reaction [36,59]. When the prey faces with a predator it will first attempt to escape through directed-oriented running. If, however, the fleeing animal is cornered so that escape is diminished, it may run blindly, without a directed orientation, or it may attempt to fight. At the moment of physical contact, often before injury is actually inflicted, the animal abruptly appears to go dead. It not only appears dead, but its autonomic physiology undergoes a widespread alteration and reorganization. The animal is in fact highly activated internally, even though outward movement is almost nonexistent. Prey animals are immobilized in a sustained (cataleptic–catatonic) pattern of neuromuscular activity and high autonomic and brain wave activity. It has been found that this immobility response has wide adaptive value: freezing makes prey less visible and non-movement in prey appears also to be a potent inhibitor of aggression in predators, often aborting attack–kill responses. Indeed, an immobile prey animal is less likely to be attacked. Much evidence has been presented implicating cholinergic, opioid and serotonergic mechanisms of the vPAG in the modulation of tonic immobility [59].

Whereas vPAG seems to be involved in the motor aspect of freezing behavior, which is probably controlled by descending projection from the amygdala to this region, dPAG appears to control the sensory and affective aspects of the unconditioned freezing. For example, stepwise increase in the electrical stimulation of both dPAG and vPAG produced initially alertness

and freezing. Freezing induced by electrical stimulation of the vPAG decreased with the termination of the stimulation. However, electrical stimulation of the dPAG induced a long-lasting freezing behavior that remained at high levels even after the interruption of the stimulation [81–83].

Evidence for the involvement of the dPAG in the generation and elaboration of defensive behavior has been extensively reported by behavioral, immunohistochemical and electrophysiological data (see [18,19,39,40] for reviews). Several reported results indicate that the dPAG is critically involved in the regulation of defensive reactions to impending danger stimuli [6–8]. As discussed earlier, the vigorous, undirected flight elicited by proximal danger would be related to panic attacks and the dPAG would be the critical structure for mediating this condition as its electrical or chemical stimulation mimics the effects of proximal danger stimuli [15].

Until some time ago, the defense reaction organized at the level of the defense brain system was considered the result of a cascade of events triggered by activation of amygdala by somatosensory stimuli leading to a set of autonomic, hormonal and behavioral responses organized at the level of the medial hypothalamus and the dPAG (Fig. 2). A limitation of this model was that only escape behavior was considered in studies with electrical stimulation of the dPAG. These studies showed that this defense response obtained from the dPAG was stimulus-bound. However, when we stimulate the dPAG at increasingly intensities, alertness and freezing appeared before escape. It has been found that these behaviors are preparatory responses for the escape and are mediated by the same neurochemical processes [14,18–20,78]. Notwithstanding, when these responses are elicited by dPAG chemical stimulation with microinjections of semicarbazide – a blocker of the glutamic acid synthesizing enzyme – the animals showed a cascade of events starting with

alertness, freezing and then escape behavior, which alternates with periods of tense immobility or freezing, in which the animals look as they were searching for an escape exit. After these findings, to consider the dPAG stimulation as an output center for the expression of the defense reaction is taken as nothing else than a simplistic view. It is now clear that a processing of aversive information is also occurring at this level of the midbrain tectum in animals facing with threatening situations.

The neural substrate of fear of the dorsal region of the dPAG seems to be related to PD. Evidence has been accumulating over the last years on the notion that anticipatory anxiety and PD would activate different brain neural networks. Although more studies are needed, the evidence obtained from functional neuroimaging studies using positron emission tomography (PET) so far tends to support this view. The first study of this kind showed activation of the whole brain aversion system during lactate-induced panic attacks, including anterior temporal lobe, amygdala and the dPAG [70,71]. It has been reported that cortical structures such as the cingulate cortex are activated during the anticipatory anxiety and this cortical structure is not activated by stimulation of the dPAG, a finding also reported during a natural, unexpected panic attack [70]. Such evidence opposes the neural circuits for anticipatory anxiety and panic in so far as the first courses with cortical activation whereas the extreme fear experienced in the second condition is produced by neuronal activity in more caudal representations [79]. In this context, freezing may also be conceptualized as the response to intermediate levels of threat or to proximal threat and pain so as two different types of freezing can be elicited depending on whether the threat is distal or proximal. These types of freezing, related to moderate and intense fear, are dissociated pharmacologically. Whereas the moderate freezing is anxiolytic-sensitive, the freezing associated with intense fear is anxiolytic resistant. On the contrary, this latter type of freezing is sensitive to the panicolytic effects of SSRIs, such as fluoxetine and is supposed to be the result of the recruitment of the neural substrates of aversion at the dPAG level [74,75].

In a similar vein, it has been shown that the interruption of the electrical stimulation of the dPAG at the escape threshold gives way to another kind of behavior. This is the so-called dPAG post-stimulation freezing. During this freezing response the dPAG and the laterodorsal nucleus of the thalamus are concomitantly activated, which suggests that the information is going up to rostral brain structures instead of being the result of activation structures associated with the motor output [13,34]. In the beginning of this century, William James and Carl Lange, and more recently Damasio [23] drew our attention to the fact that the detection of the emotional stimulus automatically (without conscious participation) produces a series of autonomic and behavioral responses which in turn provide the feedback that defines a particular emotion. The meaning of the stimulus plays an important role in the cognitive assessment we make of the dangerous situations [76]. The physiological arousal triggered by the stimulus causes unique bodily sensations and the corresponding emotional feeling with its unique quality. Together, both the cognitive assessment of the aversive stimulus and the physiological activation act in concert to produce our emo-

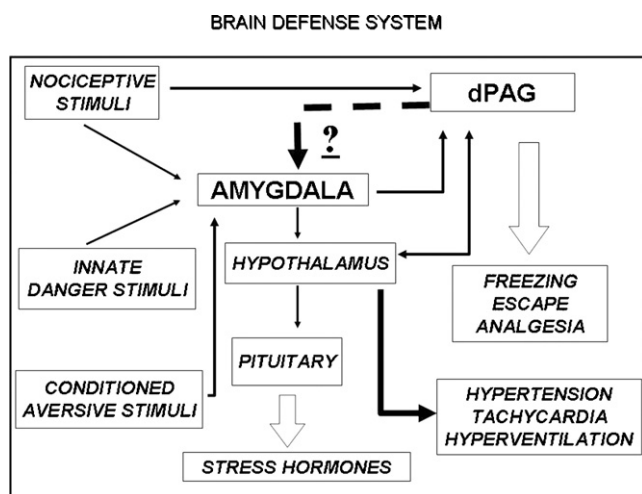


Fig. 2. The amygdala, medial hypothalamus and the dorsal periaqueductal gray (dPAG) together make part of the brain aversion system. The current notion is that they act in concert to produce the whole expression of the defense reaction with its behavioral, autonomic and endocrine components. The dPAG is thought to be the main output center for the defense reaction. However, the dPAG also processes sensory information. Thus, the question that arises is how the amygdala regulates distinct types of defense such as the dPAG-evoked freezing, the dPAG post-stimulation freezing and the conditioned freezing.

tions. Based on these principles, we can speculate that despite their overt similarity, dPAG-evoked freezing and the dPAG post-stimulation freezing are related to distinct behavioral defensive systems. The initial freezing posture triggered by dPAG electrical stimulation seems to be a preparatory response for flight. Conversely, the dPAG post-stimulation freezing allows the animal to evaluate the consequences of this aversive stimulation by brain structures located rostrally. Indeed, stimulation of the dPAG at intensities causing freezing behavior causes simultaneous Fos-labeling of the laterodorsal nucleus of the thalamus, indicating that this structure is also involved in the sensorimotor gating activated by emotional stimuli at this midbrain level [13,34]. These results suggest that whereas the vPAG is involved in the motor aspect of freezing response through descending projections to the spinal cord, the dPAG activates this defensive posture through ascending projections to rostral structures related to sensory and affective processing of aversive stimuli.

The dPAG post-stimulation freezing behavior is not context-dependent, in contrast to conditioned freezing, in which the temporal association between the environment and the unconditioned aversive stimulus is readily acquired [52,81,82]. The latter, but not the former, was abolished by lesions of the vPAG [83]. Thus, conditioned and unconditioned freezing seem to have different neural substrates, which may be related to anticipatory anxiety and panic, respectively. Whereas the first is supposed to be associated with the behavioral inhibition system/Blanchard's first and second level of defense/Bolles and Fanselow's fear-motivational system, the unconditioned freezing triggered by dPAG stimulation might be related to the Blanchard's third level of defense/Bolles and Fanselow's pain-motivational system. Going one step further, in our view the dPAG stimulation-produced freezing should be divided in two components: the pre-escape freezing derived from the activation of the neural substrates of aversion should be considered a preparatory response for the escape and the post-stimulation freezing would also be an unconditioned response, but differs from the first in that it is not an output process. Instead, it is a response to the incoming aversive information that reaches the midbrain tectum. The most remarkable difference between the dPAG-evoked response and the post-stimulation freezing is that single stimulation of the dPAG hardly acts as unconditioned stimulus in conditioning procedures whereas repeated dPAG stimulation (allowing the post-stimulation freezing) can easily be used as unconditioned stimulus in context fear conditioning [22]. Similarly, whereas panic attacks may not turn into a severe psychiatric disorder, PD characterized by recurrent panic attacks – either unexpected or associated with particular situations and persistent concern about having another attack or worry – is not easily managed by conventional therapies. In fact, it has been reported that treatments are highly successful when measured in terms of the rate of panic attack blockade, but blocking panic attacks is only part of the solution to PD. The same holds true for the PD, without and with agoraphobia, that is, when panic attacks are repeatedly associated with specific contexts the PD turns into a more severe disorder, PD with agoraphobia. This would explain the greater resistance of PD with agoraphobia to the conventional anxiolytic therapies. The reported pharmacological dissociation between

the dPAG-evoked freezing and the dPAG post-stimulation freezing gives further support to the notion that distinct biological substrates underlie these defensive reactions.

## 6. Serotonin modulation

Based on the evidence showing that the basolateral amygdala (BLA) is critically involved in the regulation of innate and conditioned reactions to threatening stimuli [24,25,53], the question that arises is whether telencephalic structures regulate the ascending information coming from the dPAG. Indeed, attenuation of freezing after activation of the neural substrates of aversion in the dPAG with inactivation of BLA supports the existence of a dPAG–amygdala loop [72,73]. We have been investigating the BLA as a probable regulator of the unconditioned and conditioned responses organized in the dPAG. A series of experiments has been conducted, in which intracerebral drug administration has been combined with electrical stimulation through the use of a permanent electrode and cannula located inside the dPAG or BLA. For determining the aversive threshold, rats were placed inside an enclosure and electrical current was applied to the dPAG with gradually increasing intensities until the rat start to run. The difference between the post-drug and the basal threshold measured the drug effect. In a recent paper we have examined the effects of the inactivation of the BLA (by enhancing its GABAergic inhibitory tone with local injections of the GABA agonist muscimol) on conditioned and unconditioned fear elicited by electrical stimulation of the dPAG. We have found that intra-BLA muscimol decreased the acquisition of fear conditioning and the dPAG post-stimulation freezing [72]. Interestingly, electrolytic or local injections of muscimol into the BLA did not change the freezing and escape thresholds determined by stepwise increases in the current of the electrical stimulation of the dPAG [65,72]. Based on this evidence, we suggested that distinct modulatory mechanisms in the BLA are recruited during the conditioned and unconditioned fear triggered by activation of the dPAG.

Once established that BLA regulates a specific type of unconditioned fear generated by stimulation of the dPAG we were also interested to find out whether the proposed connection dPAG–amygdala would also be modulated by 5-HT-mediated mechanisms since this biogenic amine has also been extensively studied in the neurobiology of fear and anxiety [27,40,41]. Using the dPAG stimulation procedure described above, we found that injections of the 5-HT<sub>2</sub> agonist  $\alpha$ -methyl-5-HT into this midbrain tectum structure caused an antiaversive effect whereas the 5-HT<sub>2</sub> antagonist ketanserin was effective in increasing the aversiveness of the dPAG electrical stimulation in rats placed in the same context in which they had previously received footshocks, but not in rats tested in a context different from where they received footshocks (Fig. 3A). Thus, the usual defensive reaction generated by simple stimulation of the dPAG in naïve animals (different context) is shifted to a distinct defense response mode when rats are placed in a context where they had experienced past stressful experience. In this latter case, there is a down regulation of 5-HT mechanisms in the dPAG which is counteracted by 5-HT agonists [67]. In contrast, it is unlikely the involvement

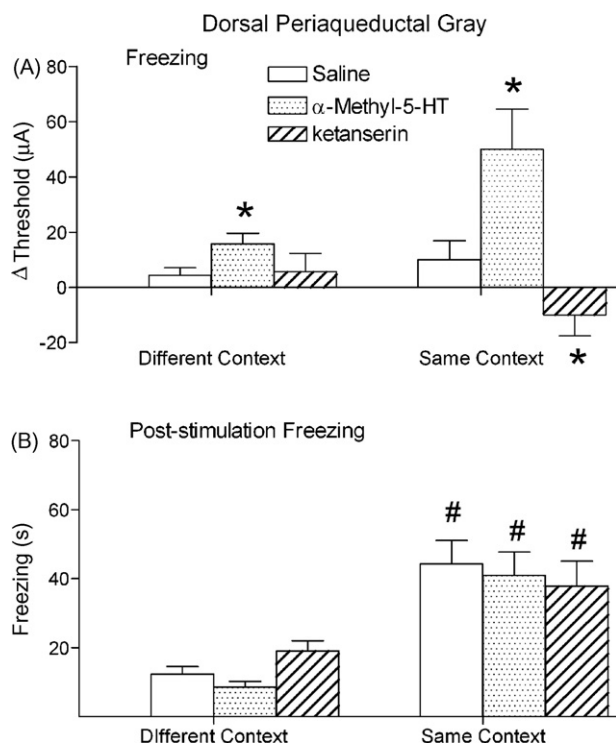


Fig. 3. (A) Mean differences ( $\Delta$ ) in the freezing thresholds determined before and after microinjections of saline,  $\alpha$ -methyl-5-HT or ketanserin into the dPAG of rats under a contextual conditioning procedure. (B) Mean of time of dPAG post-stimulation freezing after injections of saline,  $\alpha$ -methyl-5-HT or ketanserin into the dPAG of naïve, non-conditioned rats (different context) or in animals previously submitted to a contextual conditioning procedure (same context). \* $p < 0.05$  in relation to the saline group. # $p < 0.05$  in relation to the corresponding group in the different context. Modified from Ref. [67].

of 5-HT mechanisms in the processing of ascending aversive information as the post-stimulation freezing was resistant to the effects of local injections of the  $\alpha$ -methyl-5-HT and ketanserin into the dPAG (Fig. 3B). It seems that to exert its regulatory role on unconditioned fear, 5-HT<sub>2</sub> mechanisms activate GABAergic interneurons at this midbrain level [17,40,41,71]. As activation of 5-HT<sub>2</sub> mechanisms produces increased firing rate of these cells they are supposed to act through activation of the inhibitory GABAergic interneurons.

5-HT<sub>1A</sub> mechanisms are also involved in the control of defensive behaviors organized in the dPAG [28,64,86]. However, different from the 5-HT<sub>2</sub> mechanisms, they are supposed to reduce the activity of a facilitatory process since injections of 5-HT<sub>1A</sub> agonists into the dPAG causes a reduction of the firing of the cells containing these receptors [17,43]. It is suggested that in contrast with the regulatory role of 5-HT<sub>2</sub> mechanisms through GABAergic mechanisms on the output neurons of defense in the midbrain tectum, 5-HT<sub>1A</sub> mechanisms appear to act reducing the response of dPAG neurons excited by the incoming aversive stimuli [17]. These mechanisms may operate on the defense processes generated in the dPAG in a fashion similar to the way on- and off-cells regulate the nociception in the ventral PAG, activating and deactivating, respectively, the nociceptive transmission [35]. Similarly, while on-cells are excited by aversive stimuli off-cells are inhibited by these stimuli. Thus,

in terms of descending influence of the output pathways for the defensive responses, the activity of off-cells may be related to suppression of aversive transmission whereas on-cells facilitate aversive transmission. Therefore, it is likely that 5-HT<sub>2</sub> receptors activate an excitatory input to off-cells whereas 5-HT<sub>1A</sub> receptors activate an inhibitory input to the on-cells. GABAergic neurons could represent the pool of off-cells of the dPAG. The injection of the GABA receptor antagonist bicuculline into this region causes fear while injection of an agonist of the GABA-benzodiazepine receptors produces the opposite effects, i.e. antiaversive effects [15]. The excitation of the latter cells by a process of enhanced inhibition (5-HT<sub>2</sub> agonists) leads to reduction of fear. On the other hand, excitatory amino acids neurons would constitute the pool of on-cells in the dPAG and 5-HT<sub>1A</sub> mechanisms would inhibit them counteracting the fear-related pathways. Indeed, this hypothesis agree with reported evidence showing that intra-dPAG administration of the 5-HT<sub>1A</sub> agonist 8-OH-DPAT attenuated the escape behavior induced by local injection of the excitatory amino acid D,L-homocysteic acid into

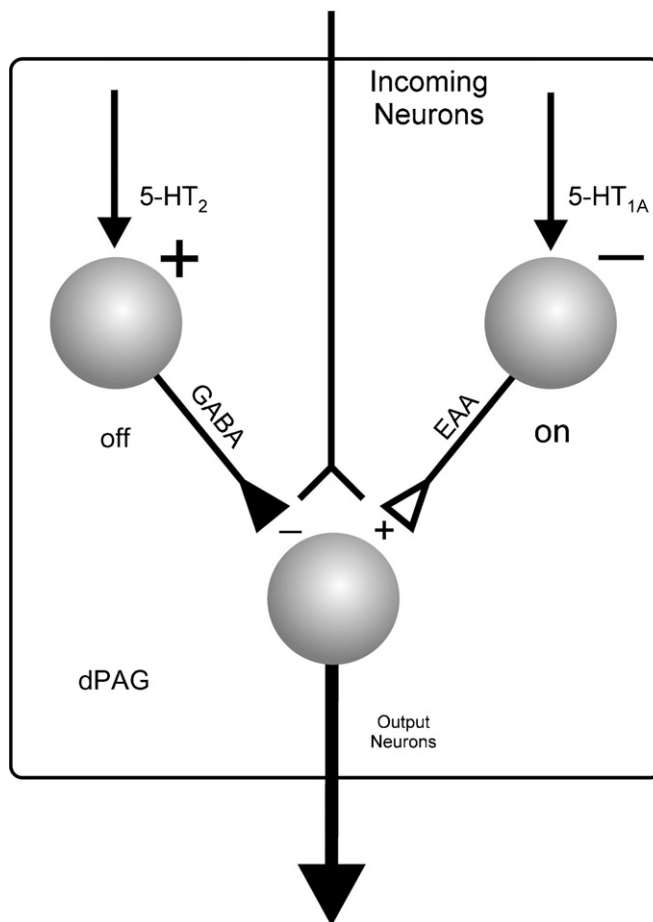


Fig. 4. Defense modulating neurons in the dorsal periaqueductal gray (dPAG). Both off- and on-cells are intrinsic neurons of the dPAG, where they exert a dual control over output neurons; on-cells excite and off-cells inhibit these neurons. Excitatory amino acids and GABA could be the neurotransmitter of on- and off-cells, respectively. While on-cells are excited by 5-HT<sub>2A</sub> agonists, off-cells are inhibited by 5-HT<sub>1A</sub> agonists. As a result both 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> mechanisms cooperate in the regulation of the neural substrates of fear in the dPAG. EAA = excitatory amino acids.



the dPAG [5]. A schematic diagram representing the interaction of these modulatory influences on the output neurons responsible for the expression of the defense reaction is depicted in Fig. 4.

To study the functional role of the BLA in the unconditioned fear generated by activation of the neural substrates of aversion in the dPAG, injections of the selective serotonin reuptake inhibitor (SSRI) fluoxetine into this nucleus were also performed in rats submitted to the dPAG electrical stimulation procedure [73]. The injections of this SSRI into the BLA did not change the aversive thresholds determined by the dPAG stimulation. The lack of change in the thresholds for freezing and escape induced by electrical stimulation of the dPAG with microinjections of fluoxetine into amygdaloid nuclei is probably linked to the fact that these unconditioned behaviors were generated by direct stimulation of the dPAG efferent downstream of the amygdala as had already been suggested by Ruiz-Martinez et al. [72]. Thus, stimulating a structure closer to the motor output, as is the case for the dPAG, overrides influences from upstream structures. Moreover, it was also found that the freezing behavior that persists after the interruption of the dPAG is sensitive to fluoxetine injections into the BLA indicating that a functional dPAG–amygdala connection seems to be activated during the processing of aversive information (Fig. 5). Taken together these studies report an opposite mediation by 5-HT mechanisms of the expression of unconditioned fear generated by dPAG stimulation and of the learning process associated with these aversive stimulations. This sums up the evidence for a pharmacological dissociation between the conditioned fear organized in the BLA and the expression of emotional behavior organized in the dPAG. Indeed, whereas local injections of fluoxetine into the dPAG cause clear antiaversive effects on the electrical stimulation of this region [12] their intra-BLA injections enhance the freezing behavior measured by a context conditioning test (Fig. 5).

One possibility that has been put forward to explain the differential role of the BLA in conditioned and unconditioned fear is that serotonergic systems may be called into play in the setting up of adaptive responses aimed at coping with or signaling the presence of threatening stimuli. Thus, the signal of the modulatory 5-HT mechanism on defensive behavior will depend on the type of emotional stimulus triggering the coping reaction. Consonant with such view, excessive functioning of 5-HT mechanisms in the BLA has been related to general anxiety disorders whereas the beneficial effects of SSRIs on panic attacks are thought to be the result of the depressive action of 5-HT on the activity of the brain aversion system [12,40,41].

Altogether, the present data indicate that the 5-HT-mediated mechanisms of the BLA appear to have opposite influences on the conditioned and unconditioned fear. While these mechanisms in the BLA appear to facilitate the conditioned fear, they inhibit the unconditioned fear triggered by activation of the dPAG. Evidence is presented in support of the hypothesis that whereas dPAG-evoked freezing would serve as a model of panic attacks, the dPAG post-stimulation freezing appears to be a model of panic disorder. It is also proposed that conditioned freezing plus dPAG electrical stimulation procedure might also mimic panic disorder with agoraphobia. It has been

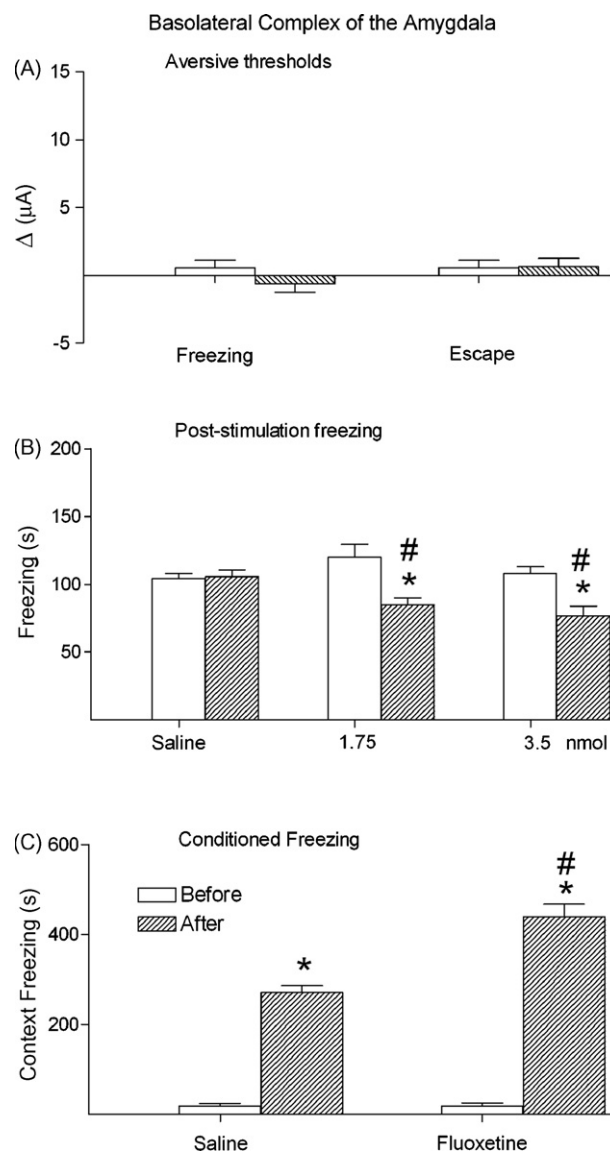


Fig. 5. (A) Mean differences ( $\Delta$ ) in the freezing thresholds determined by the dPAG stimulation procedure before and after microinjections of saline and of fluoxetine into the basolateral complex of the amygdala. (B) Mean of time of dPAG post-stimulation freezing after injections of saline or fluoxetine into the basolateral complex of the amygdala. (C) Mean of time of freezing after injections of saline and fluoxetine into the basolateral complex of the amygdala in animals submitted to a contextual conditioning procedure. \* $p < 0.05$  in relation to the control. # $p < 0.05$  in relation to the saline group. Modified from Ref. [73].

shown that the dPAG post-stimulation freezing is related to the process of ascending information to higher centers probably to the amygdala and seems to be associated with the component of avoidance and uncertainty characteristic of the PD. 5-HT uptake inhibitors also attenuate these responses by acting on 5-HT neurons of the BLA where they also enhance the inhibitory function of neuronally released 5-HT and decrease the symptoms of PD. Therefore, it is conceivable that a deficiency of 5-HT modulation of the brain aversive system may be involved in the triggering of PD. From these data it has been suggested that a deficit in 5-HT inhibition at the level of the dPAG may underlie the susceptibility to panic attacks that char-

Table 1  
Different patterns of freezing associated with distinct types of anxiety

Procedure	Distinct patterns of freezing behavior								Associated anxiety disorder
	Associative learning	Piloerection and exophthalmus	dPAG	Amygdala	GABA neurons of amygdala	5-HT neurons of amygdala	HPA involvement	Fear-potentiated startle	
Context fear conditioning (CFC)	Yes <sup>a</sup>	No <sup>b</sup>	Yes <sup>c</sup>	Yes <sup>d</sup>	Decrease <sup>e</sup>	Increase <sup>f</sup>	Yes <sup>g</sup>	Yes <sup>h</sup>	Generalized anxiety disorder
Electrical stimulation of the dPAG	No <sup>i</sup>	Yes <sup>j</sup>	No <sup>k</sup>	No <sup>l</sup>	?	?	No <sup>m</sup>	?	Panic attacks
dPAG post-stimulation freezing	No <sup>n</sup>	?	?	Yes <sup>o</sup>	Decrease <sup>o</sup>	Decrease <sup>p</sup>	?	?	Panic disorder without agoraphobia
CFC with dPAG stimulation as UCS	Yes <sup>q</sup>	?	?	?	?	?	?	?	Panic disorder with agoraphobia

dPAG: dorsal periaqueductal gray; CFC: contextual fear conditioning; HPA: hypothalamic–pituitary–adrenal axis; ?: lack of data.

<sup>a</sup> Fanselow [31].

<sup>b</sup> Bolles and Collier [9].

<sup>c</sup> Kim et al. [50].

<sup>d</sup> Phillips and LeDoux [68].

<sup>e</sup> Muller et al. [62].

<sup>f</sup> Inoue et al. [44].

<sup>g</sup> Pugh et al. [69].

<sup>h</sup> Davis [24].

<sup>i</sup> Vianna et al. [82].

<sup>j</sup> Brandão et al. [15].

<sup>k</sup> Vianna et al. [83].

<sup>l</sup> Oliveira et al. [65].

<sup>m</sup> Schenberg et al. [77].

<sup>n</sup> Vianna et al. [81].

<sup>o</sup> Ruiz-Martinez et al. [72].

<sup>p</sup> Ruiz-Martinez et al. [73].

<sup>q</sup> Di Scala et al. [30].

acterizes PD. Conversely, intensification of 5-HT inhibition in the dPAG by anti-depressants may be a mechanism of the anti-panic action of these compounds. Together, these data support the two-dimensional view of defense in that anxiety and fear are represented in parallel systems in the brain, which are probably modulated by opposing neurochemical mechanisms [57].

## 7. Conclusions

Animal models are important developments in investigations of the mechanisms underlying a human disease and the design of new treatments. The biological fidelity of some aspects of new models of psychiatric disorders, particularly anxiety, has generated new evidence about the neurobiology and has been useful in providing new insights about the pathophysiology of this condition. In this context, much evidence has been accumulated over the last years indicating that the neural circuits for anticipatory anxiety and panic are distinct. The reviewed evidence indicates that these disorders are different not only in terms of subjective experience, but also in behavioral and physiological manifestations, response to drugs and neural substrates. Anxiety is associated with defensive reactions to potential threat, attenuated by anxiolytic drugs, integrated in limbic forebrain structures such as the amygdala and the hippocampus, and activates the HPA hormonal axis. On the other hand, panic is related to defense reactions elicited by proximal threat, resistant to anxiolytics, integrated in primitive structures of the hindbrain, such as the PAG, and does not activate the HPA axis. Whereas the first courses with cortical activation, the extreme fear experienced in the second condition is produced by neuronal activity in more caudal representations.

The present review presents a series of evidence relating different defensive patterns with specific anxiety disorders. In this context, there are at least four different kinds of freezing with specific neural substrates. The immobility induced by stimulation of the ventral column of the periaqueductal gray has been considered a quiescence characteristic of the recovery component of defense–recuperative processes. There is an isomorphism between freezing response to contextual stimuli paired with electrical shocks and generalized anxiety disorder. Besides, two types of freezing emerge with the electrical stimulation of the dorsal aspects of the periaqueductal gray (dPAG): the dPAG-evoked freezing and the dPAG post-stimulation freezing. These two conditions should be considered in the context of the differences between panic attacks and PD discussed earlier in this review. Table 1 summarizes the main behavioral, autonomic, hormonal and neural circuits differences that have been reported in the literature for the conditioned freezing, dPAG-evoked freezing and dPAG post-stimulation freezing. The amount of question marks in this table gives the real dimension of what is still needed to be done to better characterize the different types of freezing and to validate the model thoroughly. The combined use of the dPAG stimulation procedure and drug injections into the amygdaloid nuclei and in the dPAG itself can be a useful tool in understanding the differences in panic attacks, panic disorder with and without agoraphobia and in understanding the differences in the effects of anxiolytic med-

ications with distinct mechanisms of action. Finally, based on several studies conducted in this and other laboratories, it is proposed that off- and on-cells of the neural substrates of fear generated at the level of the dPAG are modulated by 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> mechanisms, respectively. The understanding of how the periaqueductal gray generates and elaborates different types of freezing is of relevance for our better knowledge of distinct types of anxiety.

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