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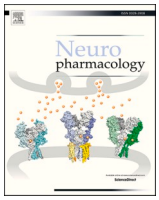

Orchestration of innate and conditioned defensive actions by the periaqueductal gray

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ABSTRACT

The midbrain periaqueductal gray (PAG) has been recognized for decades as having a central role in the control of a wide variety of defensive responses. Initial discoveries relied primarily on lesions, electrical stimulation and pharmacology. Recent developments in neural activity imaging and in methods to control activity with anatomical and genetic specificity have revealed additional streams of data informing our understanding of PAG function. Here, we discuss both classic and modern studies reporting on how PAG-centered circuits influence innate as well as learned defensive actions in rodents and humans. Though early discoveries emphasized the PAG's role in rapid induction of innate defensive actions, emerging new data indicate a prominent role for the PAG in more complex processes, including representing behavioral states and influencing fear learning and memory.

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1. Introduction

The midbrain periaqueductal gray (PAG) is conserved across all vertebrate species, and is a structure known to have a central role in various motivated behaviors, including appetitive drive, sexual behavior, and defensive responses. The role of the PAG in defensive behaviors has been investigated for decades. Rodent tracing studies have indicated that the PAG is anatomically uniquely-well-suited to mobilize defensive actions, as it receives inputs from a constellation of structures known to be activated by threats, including the prefrontal cortex (PFC) and anterior cingulate area (ACA), the anterior hypothalamus, the superior colliculus, the dorsal premammillary nucleus (PMd) and the central amygdala, among others (Gross and Canteras, 2012; Silva and McNaughton, 2019). Though PAG afferent connections have been studied in more detail, it is important to note that bottom-up PAG projections have been observed to the anterior hypothalamus, thalamus, zona incerta and central amygdala, indicating that the PAG also influences forebrain structures (Motta et al., 2017). Similar connectivity is thought to also exist in humans, as several studies using diffusion tractography imaging show that the PAG is structurally connected to, among other regions, thalamus, hypothalamus, amygdala, ventromedial (vmPFC) and ventrolateral prefrontal cortex (Hadjipavlou et al., 2006; Owen et al., 2008; 2007: Pereira et al., 2010; Sillery et al., 2005). Further, functional connectivity using a PAG seed during a threat imminence task, showed increased coupling with the amygdala, vmPFC, mid-ACA and insula (Mobbs et al., 2009), and for fast escape responses, the PAG was coupled with mid-ACA and thalamus (Qi et al., 2018), indicating that in humans the PAG participates in distinct functional networks contingent on the imminence of threat.

A large stream of data from anatomical and functional activation studies revealed that the PAG may be divided into different columns. Such as the dorsomedial and dorsolateral (dorsal PAG), lateral, and ventrolateral columns. Each of these PAG subregions is characterized by distinct afferents, efferents (i.e., ascending prosencephalic targets) (Motta et al., 2017), and molecular profiles (Bandler and Keay, 1996; Carrive, 1993; Silva and McNaughton, 2019). From this perspective, the complexity of the defensive actions organized at the midbrain level can be partly understood based on the heterogeneity of PAG inputs (Fig. 1).

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The PAG receives dense afferent connections from the frontal cortex, temporal areas, the hypothalamus, the zona incerta, tectum, the midbrain and the medulla (Silva and McNaughton, 2019). Through its afferent inputs from aversive sensory systems and the forebrain, the PAG is in a position to provide an emotional primary process and may be critical in coordinating defensive behavioral responses to unconditioned and learned aversive events.

The central nucleus of the amygdala is the only amygdaloid structure that projects to the PAG, innervating both the ventral and dorsal portion of the PAG (Rizvi et al., 1991). This nucleus contains distinct and mutually inhibitory neuronal populations that exert bidirectional control over conditioned freezing and flight (Fadok et al., 2017). Furthermore, projections from the central nucleus to the ventrolateral PAG control the expression of conditioned freezing (Tovote et al., 2016).

Although the PAG does not receive direct projections from the hippocampus or septum, the perirhinal and entorhinal cortices send inputs to the dorsal PAG (Witter and Groenewegen, 1986a; 1986b) play an important role in the formation of contextual fear memories (Basu et al., 2016; Bucci et al., 2021; Kitamura et al., 2017, 2015). In contrast, the ventrolateral PAG receives inputs from the habenula, a brain region that is involved in regulating anxiety, fear and reward. The habenula is also known to encode aversion (Szőnyi et al., 2019; Trusel et al., 2019), and can impact the formation of contextual and cued fear memories that could be critical in fear generalization (Sachella et al., 2022). Future studies are needed to determine how habenular input to the PAG affects these behaviors.

The zona incerta, an integrative node for sensory processing, motivational drive, and behavioral output control (Fratzl and Hofer, 2022; Wang et al., 2020), sends extensive projections to the PAG that influence arousal, object discrimination, novelty exploration (Ahmadlou et al., 2021), appetitive drive (Zhao et al., 2019), and fear (Chou et al., 2018; Li et al., 2021; Wang et al., 2019; Venkataraman et al., 2019).

The role of the PAG in mediating fear is also substantially influenced by hypothalamic activity (Gross and Canteras, 2012). All columns receive dense projections from the anterior and the ventromedial hypothalamus, while the lateral hypothalamus projects more heavily to the ventrolateral PAG, exerting bidirectional control over approach and avoidance (Li et al., 2018). Direct activation of the anterior hypothalamus elicits avoidance and escape jumping, but not immobility (Wang et al., 2015), and its projections to the ventrolateral PAG are sufficient to promote defensive attack towards predators (Xie et al., 2022). The ventromedial hypothalamic area activation (which is reciprocally connected with the anterior hypothalamus) induces inflexible immobility via projections to the dorsolateral PAG (Wang et al., 2015), while activation of the PMd inputs to the dorsolateral PAG coordinates the execution and vigor of escape responses (Wang et al., 2021a,b). From the midbrain tectum, excitatory inputs originating in the superior colliculus mostly target the dorsal PAG and provide a synaptic threshold for PAG activation and the initiation of escape evoked by looming stimuli (Evans et al., 2018).

The PAG also receives extensive cortical innervation. The ACA projections to the PAG exclusively targets the dorsolateral column (Wyss and Sripanidkulchai, 1984) while the infralimbic area targets predominantly the ventrolateral PAG (Hurley et al., 1991; Takagishi and Chiba, 1991). The anterior portion of the prelimbic cortex also projects more densely to the ventrolateral PAG, and its posterior area targets the dorsal portion of the PAG (Floyd et al., 2000; Sesack et al., 1989). These cortical inputs to the PAG might impact defensive states by influencing fear generalization processes (Rozeske et al., 2018), fear responses to social avoidance (Franklin et al., 2017) place aversion (Vander Weele et al., 2018) and the expression of risk assessment and contextual avoidance during exposure to predatory context (de Lima et al., 2022).

Modulatory neurotransmitters can also influence PAG activity through projections originating from the locus coeruleus towards several portions of the PAG (Jones and Moore, 1977) while the substantia nigra predominantly targets the ventrolateral PAG (Gerfen et al., 1982; Kirouac et al., 2004). Serotonergic processes can be identified in all the subdivisions of the PAG, but the ventral regions present the highest densities, with an increase toward the caudal portion of the PAG (Clements et al., 1985; Steinbusch, 1981). Lastly, the medullary sensory projections (containing adrenergic and noradrenergic projections) target almost all the PAG sub-divisions (Blomqvist and Craig, 1991; Herbert and Saper, 1992). Though the functions of these inputs are mostly unknown, prior studies indicate that they may strongly influence defensive behavior. For example, serotonergic transmission in the PAG decreases active escape (Zanoveli et al., 2003).

Recent advances in neuronal targeting techniques have revealed a complex and distinct cellular organization, functional connectivity and identified specific behavioral functions in the PAG (Vaughn et al., 2022). Recents studies have both confirmed and refined the canonical columnar architecture model of the PAG, and demonstrate that genetic diversity constitutes an additional axis of functional organization within the PAG (Deng et al., 2016; Evans et al., 2018; Gao et al., 2019; La-Vu et al., 2022; Montardy et al., 2020; Tovote et al., 2016).

Early studies revealed that electrical stimulation of the PAG in humans causes panic-related responses, including a catastrophic fear of dying (Nashold et al., 1969). The development of functional imaging methods showed that the PAG is active during both anticipation and experience of aversive responses (Faull and Pattinson, 2017; Mobbs et al., 2007). Similar results were also observed in rodents, as PAG stimulation produced protean escape and freezing in rats (Brandão et al., 2008). Decades of studies on the PAG in rodents have demonstrated that

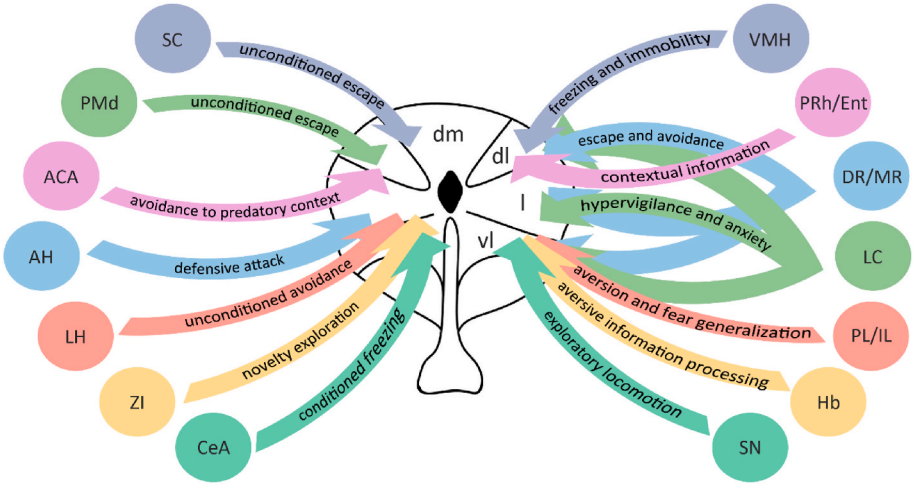

Fig. 1. Afferent projections to the PAG that influence specific aspects of defensive related behaviors. See text for discussion. Abbreviations: ACA – anterior cingulate area; AH – anterior hypothalamic nucleus; CeA – Central amygdalar nucleus; LH – lateral hypothalamic area; PMd – dorsal premammillary nucleus; SC – superior colliculus; ZI – zona incerta; VMH – ventromedial hypothalamus; PRh – perirhinal cortex; Ent – Entorhinal cortex; DR – dorsal raphe; MR – median raphe; LC – locus coeruleus; PL – prelimbic cortex; IL – infralimbic cortex; Hb – habenula; SN – substantia nigra; dl – periaqueductal gray, dorsolateral part; dm – periaqueductal gray, dorsomedial part; l – periaqueductal gray, lateral part; vl – periaqueductal gray, ventrolateral part.

the PAG is involved in the full repertoire of defensive actions, ranging from hiding to flight and defensive biting attacks. Interestingly, though the PAG is mostly studied in the context of innate defense, a growing body of literature demonstrates its involvement in learned defensive responses as well, which may recruit cortical targets via the PAG's projections to the thalamus (Krout and Loewy, 2000; Yeh et al., 2021). In parallel, recent data from human subjects also indicate a central role for the PAG in threat-induced actions.

Here, we present an up-to-date review of the evidence indicating that the PAG is part of a broad neural network that plays a complex role in the regulation of defensive states, which can be elicited by both innate as well as conditioned threats in both rodents and in humans. Through its diverse neurotransmitter systems and anatomical connectivity, which includes reciprocal connections with the forebrain (Marín-Blasco et al., 2020), the PAG exerts a role in integrating and controlling a full spectrum of multifaceted behavioral functions.

2. PAG and innate fear responses

The periaqueductal gray (PAG) has multiple complex functions. The PAG has an integral role in autonomic function, motivated behavior and behavioral responses to painful and threatening stimuli that are crucial for rapid behavioral adaptation of animals to their environment. Traditionally, the PAG has been described as an output region of a wide brain network involved in defensive responses. Accordingly, stimulation of dorsal and ventral parts of the PAG of rodents produces aversive states with different behavioral outputs (Brandão et al., 2008; Deng et al., 2016; Koutsikou et al., 2014; Tovote et al., 2016) such as escape and freezing. Current models largely view the PAG as a downstream effector region of a wide neural defensive circuit, such that computational decisions to perform a particular behavior are processed in other upstream regions in the brain, while PAG activation merely executes the defensive response and controls its vigor (Evans et al., 2018; Tovote et al., 2016). However, a growing body of evidence suggests that the PAG might have a more complex role in defensive processing. The PAG controls defensive responses which comprises not only freezing and escape, but also risk assessment and innate anxiety. It was shown that the dorsal PAG independently encodes distance to threat and numerous defensive behaviors, including freezing, stretch-attend postures, and escape (Esteban Masferrer et al., 2020; Reis et al., 2021b). In order to adapt and optimize chances of survival, during naturalistic encounters with a predator, mice can voluntarily approach and avoid the source of threat. In this condition, the dorsal PAG is activated prior to escape in face of a predator that can be predicted up to 3 s before the escape is initiated, indicating that this region may be computing optimal behavior selection, and is not merely acting as a motor output activation node (Reis et al., 2021b). This result indicates that the dorsal PAG may be involved in preparatory computations that affect the timing and the decision to escape, rather than only serving as a downstream effector region to initiate flight. By performing population-level analysis of PAG neurons, it was demonstrated that PAG cells are not only encoding specific behaviors or threat imminence, but ensemble activity reflects moment-to-moment changes in the approach-avoidance state of the animal using encoding patterns that are shared across different threatening situations (Reis et al., 2021a). Though dorsal PAG mediated escape is mostly studied in relatively simple situations, recent data indicated that coordinated activation of dorsal premammillary projections to both the dorsolateral PAG and the medial ventral thalamic nucleus is necessary in situations requiring complex context-specific escape strategies (Wang et al., 2021b). Additionally, dorsal PAG stimulation can induce persistent changes on behavioral states such as subsequent place aversion and behavioral sensitization up to 2 weeks after stimulation offset (Carvalho et al., 2018; Deng et al., 2016). Part of these long-lasting effects seems to be mediated by neurokininergic mechanisms in the amygdala (Carvalho et al., 2018). Together, these findings suggest that the PAG activity promotes and reflects the internal brain states that prepare the organism to engage in approach or avoidance of different types of threat.

The PAG function is modulated by several neurotransmitters that interact in a complex way to influence defensive behaviors (Fogaça et al., 2012). The PAG is involved in emotional processing and, hence, integrates a variety of input signals and also initiates responses to them (Wang et al., 2021b). Glutamatergic inputs are one of the most common excitatory inputs to the PAG and glutamate receptors are ubiquitously distributed throughout its columns (Tölle et al., 1993). It has been demonstrated that optogenetic activation of glutamatergic projections from the lateral hypothalamic area, the PMd, and the superior colliculus to the PAG induces escape (Evans et al., 2018; Li et al., 2018; Wang et al., 2021b). Additionally, activation of glutamatergic inputs from the ventromedial hypothalamus promotes freezing (Wang et al., 2015), while activation of GABAergic inputs from the anterior hypothalamus promotes defensive attacks and biting (Xie et al., 2022). The administration of *N*-methyl-D-aspartate (NMDA) glutamate receptor agonist in the PAG provokes the expression of unconditioned defensive behaviors (Bertoglio and Zangrossi, 2006; Carrive, 1993). Additionally, escape responses can be induced by intra-dorsal PAG administration of a group I and II metabotropic glutamate receptors (mGluR) agonist (Lima et al., 2008). By contrast, intra-dorsal PAG administration of NMDA and non-NMDA receptor antagonists decreases anxiety-related responses in the elevated plus maze (Guimarães et al., 1991; Matheus and Guimarães, 1997; Molchanov and Guimarães, 2002). Accordingly, neurons expressing the vesicular glutamatergic transporter (VGLUT2) in the dorsal PAG encode the choice to escape and can control escape vigor in response to innately aversive overhead looming stimulus (Evans et al., 2018). Conversely, activity of ventrolateral PAG VGLUT2-expressing cells are necessary for both learned and innate freezing responses (Tovote et al., 2016).

In comparison, anti-aversive effects are observed with the administration of γ-amino-butyric-acid (GABA) GABA-A receptor agonist muscimol or benzodiazepine receptor agonists. The inhibitory effects mediated by GABA can attenuate the aversive effects induced by dorsal PAG electrical stimulation (Graeff et al., 1986), which has been proposed as a model of panic attack (Schenberg, 2010). The role of GABA in modulating defensive responses can be exemplified by the effects observed after intra-PAG administration of GABA-A antagonists or the inhibitor of glutamic acid decarboxylase (the enzyme that catalyzes the conversion of glutamate to GABA) that promote unconditioned flight reactions similar to those observed during proximal threatening situations (Brandao et al., 1986; Graeff et al., 1986; Perusini and Fanselow, 2015; Schmitt et al., 1985). This pharmacological evidence suggests the existence of GABAergic mechanisms tonically inhibiting the PAG circuit that modulates defensive responses. Accordingly, the optogenetic inhibition of ventrolateral PAG GABAergic GAD2-expressing neurons is sufficient to induce freezing (Tovote et al., 2016). Convergently, the benzodiazepine receptors in the PAG are a potential target for the anxiolytic effects induced by systemically injected benzodiazepines (Russo et al., 1993).

In contrast, whereas the micro infusions of 5-hydroxytryptamine-1A (5-HT1A) or 5-HT2 receptors agonists in the dorsal PAG can produce anxiolytic-like effects in animal models of anxiety, the 5-HT receptor blockers failed to produce aversive behavior per se (de Paula Soares and Zangrossi, 2004; Graeff et al., 1986). More importantly, 5-HT receptor agonists and systemic fluoxetine administration mitigate the aversive consequences of dorsal PAG electrical stimulation (Borelli et al., 2004; Graeff et al., 1986). These effects have similarities between the clinical pharmacological response to SSRIs treatment which are known to inhibit panic-like responses (Batelaan et al., 2012; Pollack et al., 1998).

The actions of other neurotransmitters (such as those mediated by neuropeptides) in the PAG also contribute to the modulation of aversive states. Microinjections of cholecystokinin (Netto and Guimarães, 2004), corticotropin-releasing hormone (Litvin et al., 2007) or substance P (Aguiar and Brandão, 1996) increases anxiety-like responses. The atypical neurotransmitters nitric oxide and the endocannabinoids,

which are produced 'on demand' and released from the postsynaptic neuronal membrane, can also exert modulatory functions in the PAG by interacting with other neurotransmitter systems and impact the emotional states (Fogaça et al., 2012). For example, exposure to the elevated plus maze (Beijamini and Guimarães, 2006a) or a predator (Aguiar and Guimarães, 2009; Beijamini and Guimarães, 2006b) is sufficient to activate NOS-positive neurons in the dorsolateral PAG. Intra-dorsal PAG injections of drugs that decrease NO activity decreases defensive behaviors in both behavioral paradigms (Aguiar and Guimarães, 2009; Guimarães et al., 1994) while the administration of NO donors can induce escape responses (Braga et al., 2009; de Oliveira et al., 2000). By contrast, local administration of low doses of the cannabinoid receptor agonist anandamide, through activation of CB1 receptors, can attenuate defensive behaviors by decreasing glutamate release (Moreira et al., 2007; Vaughan et al., 2000). Thus, the heterogeneity of its neuronal populations and the different functions of several neurotransmitter systems interact in a complex way to control emotional states and defensive responses in this region.

Overall, the diversity of the PAG neurotransmitter systems could indicate that genetically-identified cell populations of PAG may have specific roles in fear and anxiety (Fig. 2). Recently, few studies have described new neural mechanisms responsible for specific functions orchestrated in the PAG. It was demonstrated that the optogenetic activation of VGLUT2 neurons or the inhibition of a specific gabaergic neuronal population in the ventrolateral PAG is sufficient to promote freezing behavior (Tovote et al., 2016). In contrast with the classical view that the dorsal part of the PAG is predominantly responsible for the occurrence of escape events, and that the ventrolateral PAG mediates freezing behaviors, La-Vu et al. showed that activation of lateral and ventrolateral PAG cholecystokinin-expressing cells selectively caused active coping strategies (escape) to safer regions within an environment (La-Vu et al., 2022), while pan-neuronal activation of the same area created freezing. Specific neural inputs from VGLUT2 cells originating in the lateral hypothalamic area towards the PAG supports the existence of a defensive circuit that engages the lateral and ventrolateral PAG to promote evasion and unconditioned aversion (Li et al., 2018). Supporting the view that the molecular identity and connectivity of PAG neurons might have specific roles to integrate information processing and encoding aversive states, activation of a subset of glutamatergic cells expressing Tac-1 in lateral and ventrolateral PAG have a multimodal role controlling both itch-scratching responses and anxiety-related behaviors (Gao et al., 2019). Other unstudied PAG populations may have similarly interesting roles in behavioral adaptation. For example, a recent study using calcium imaging by fiber photometry recorded the activity of VGLUT2+ and GAD2+ neuronal populations in the dorsal PAG during unconditioned and conditioned aversive stimulation. The results suggest that both VGLUT2 and GAD2 neuronal populations respond to direct unconditioned stimulus (US), and to conditioned stimulus (CS) presentations during conditioning, but only the dorsal PAG VGLUT2+ population showed persistent response to the CS stimulation during retrieval (Montardy et al., 2020). These data

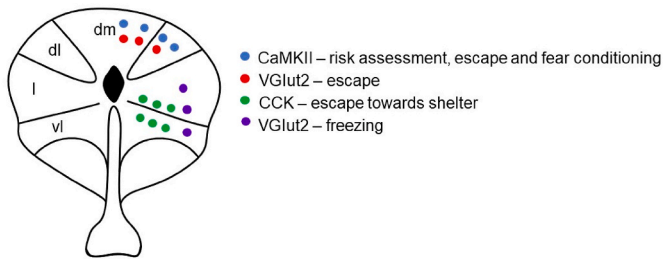

Fig. 2. Defensive functions identified in specific neuronal populations in the PAG. Abbreviations: dl – periaqueductal gray, dorsolateral part; dm – periaqueductal gray, dorsomedial part; l – periaqueductal gray, lateral part; vl – periaqueductal gray, ventrolateral part.

underscore the importance of dissecting the function of PAG neurons defined by genetic identity.

Indeed, recent results show that different genetically identified PAG cells are activated during distinct behaviors (Vaughn et al., 2022), indicating that genetic diversity is a key unexplored functional organization axis within the PAG. This study provided a transformative framework for future anatomical, molecular and functional explorations of the PAG. In this work, by using snRNA-seq and spatially resolved single-cell transcriptomic measurements (MERFISH), it was possible to map transcriptional and behavioral features in individual cells of the PAG. Vaughn et al. (2022) revealed three-dimensional spatial motifs in neuronal cell types, and identified neuromodulatory gene expression patterns in more than 100 clusters across excitatory or inhibitory neurons in the PAG. The results obtained with this approach refine, revise and redefine the previous coarse anatomical subdivisions of the PAG across its columns. For example, some of the identified clusters agreed with the classical PAG columnar organization and described in detail the cellular composition of the structures. Other clusters refined the existing columnar organization by identifying specific cell populations more densely expressed in the inner and outer portion of the PAG structure. Lastly, the analysis revealed spatial motifs that do not agree with the classical PAG columnar description. On top of that, within individual clusters, the neural activation may show substantial variability along its rostrocaudal axis across different behavioral states. After predator exposure, the activated neuronal clusters presented enrichment of the orexin receptor orexin receptor genes HCRTR1 and HCRTR2, the corticotropin releasing hormone receptor CRHR1, and the alpha-adrenergic receptor gene ADRA1a, and the dorsolateral PAG neuronal population marked by PAX7 and TFAP2B showed very specific activation. Thus, with more experiments performed using advanced molecular techniques, further studies may reveal new contributions of these newly defined spatial compartments, complementing and revising the existing anatomical and functional delineations of the PAG.

The vast majority of studies that investigated the role of PAG in defensive behaviors focus mostly on its columnar structures rather than its rostral-caudal axis organization. However, different neuroanatomical connections (Chen and Aston-Jones, 1996; Rizvi et al., 1991, 1992; Silva and McNaughton, 2019) and gene expression along the longitudinal axis of the PAG (Gao et al., 2019; La-Vu et al., 2022; Tovote et al., 2016; Vaughn et al., 2022) might have distinct functions. For example, studies using chemical stimulation of the PAG (e.g., kainic or glutamic acid) showed that strong backward defense movements (e.g., upright standing, alerting and backing) were induced mostly by stimulation of the intermediate third along the rostro-caudal PAG axis in rats (Bandler et al., 1985; Bandler and Depaulis, 1991; Depaulis et al., 1992; 1989). In contrast, the majority of sites at which strong forward defensive responses were evoked (e.g., burst of forward locomotion alternated with periods of immobility and occasional jumps) were localized within the caudal third of the PAG. Freezing (immobility) was evoked by ventrolateral stimulation, in the caudal one half of the PAG. Also, the anxiogenic effects observed in the elevated plus maze test induced by glycine injections in the dorsal PAG seems to be specific to its caudal portions (Teixeira and Carobrez, 1999). The differential receptor distribution, specific anatomical connections and the dynamic neuronal activity within different parts of the PAG during specific behavioral tasks may impact the contribution of each portion of PAG's columns. Future studies using viral vectors strategies will be able to better characterize the functional and anatomical differences of PAG portions across its longitudinal axis.

Taken together, data from various lines of evidence support the view that PAG activity controls a broad ethogram of defensive states and is not simply acting as a common downstream effector region (Marín-Blasco et al., 2020; Motta et al., 2017). Intriguingly, population-level analysis and advances in computational methods applied to neural data indicate that the PAG is performing sophisticated information processing, coordinating the encoding of internal behavioral states,

future motor actions and ongoing external stimuli (Esteban Masferrer et al., 2020; Evans et al., 2018; Reis et al., 2021a,b). Future studies investigating the precise contribution and connectivity of specific PAG cellular populations to orchestrate unconditioned defensive behaviors will open new avenues for a better mechanistic understanding of the PAG role in fear responses. Furthermore, creating more naturalistic testing conditions in the lab by evaluating stimulus processing that mimics what happens in animals facing threats in nature can gather evidence of more realistic physiological functioning mechanisms.

3. PAG and learned fear responses

Learned fear responses have been largely investigated using shock-based fear conditioning, where animals are typically confined inside the shocking chamber during the fear conditioning test. In this situation, freezing is the main learned fear response to physically harmful events, such as electric footshock (Perusini and Fanselow, 2015; Tovote et al., 2016). Based on electrical stimulation, lesion, and pharmacological studies, the PAG has been proposed to present an essential part of the circuitry that elicits freezing in response to threat (Bandler and Carrive, 1988; Carrive, 1993). Using shock-based fear conditioning, a GABAergic pathway from the central amygdala to the ventrolateral PAG seems critical for eliciting freezing (Tovote et al., 2016). In an innovative version of the shock-based fear conditioning paradigm (Rozeske et al., 2018), showed evidence that a mPFC-ventrolateral PAG neural pathwal contributes to discriminating a previously threatening context from a neutral context. At least two downstream anatomical pathways from the ventrolateral PAG have been identified to mediate freezing. The first involves a direct glutamatergic projection to premotor cells in the magnocellular nucleus in the medulla (Tovote et al., 2016), and the other path links the ventrolateral PAG to the cerebellar vermal cortex, engaging an olivocerebellar circuit terminating as climbing fibers in the lateral vermal lobule VIII, which are likely to influence motor pathways known to regulate muscle tone (Koutsikou et al., 2015; 2014).

However, classical fear conditioning studies are limited to mimicking what happens in animals facing threats in nature. Instead of being trapped in a specific threatening location, under natural conditions, animals need to perceive the threats and remember the area where the threat occurred so that the possibility of re-encountering the same threat can be avoided (Blanchard et al., 1989). Thus, a defensive response to a natural threat is a flexible cognitive process that utilizes the knowledge of prior experiences and environments.

Notably, in testing shock-based fear conditioning in a situation with free access to the conditioning compartment, animals displayed almost no freezing, avoided entering the shock chamber, and exhibited risk assessment responses which is a very complex response, including carefully scanning the environment in the crouched position (crouch sniffing) and attempting to approach the threatening stimulus by stretching the body (stretch postures) (Blanchard et al., 2011; Viellard et al., 2016). Moreover, shock-conditioned animals that could avoid the conditioning chamber engaged a distinct septo/hippocampal -hypothalamic-brainstem circuit, comprising the ventral subiculum, the juxtadorsomedial lateral hypothalamic area, the PMd, and the different columns of the PAG (Viellard et al., 2016). Photoinhibition of the pathway from the PMd to the PAG impaired risk assessment and avoidance of the conditioning chamber contextual avoidance in shock-conditioned animals tested with free access to the conditioning chamber (Viellard, 2019). As we shall discuss, it is reasonable to believe that this elaborated display of PAG-induced risk assessment is also organized by prosencephalic circuits.

In addition to shock-based fear conditioning, the PAG is involved in fear-learned responses in the environment related to predatory threats. During exposure to environments previously visited by a predator, the predatory threat is more ambiguous, and the animals present risk assessment responses (Cezario et al., 2008; Ribeiro-Barbosa et al., 2005). The memory of predatory threats is likely to be stored in the basolateral amygdala and ventral hippocampus (Reis et al., 2022) that engage the predator-responsive circuit in the medial zone of the hypothalamus (comprising the dorsomedial part of the ventromedial nucleus, the anterior nucleus and the PMd) along with the PAG during re-exposure to a context previously paired with a live predator (Gross and Canteras, 2012). Notably, pharmacological inactivation of the PMd was able to practically abolish the predatory contextual conditioned responses. This sharp decline in the contextual conditioned defense was associated with a large decrease in activation levels in the rostral half of the dorsolateral and dorsomedial PAG columns (Cezario et al., 2008). In the PAG, exposure to the predatory context yields clear Fos up-regulation, particularly in the dorsomedial and dorsolateral parts, like that has been described during direct exposure to a live predator, but less intense (Cezario et al., 2008). In line with this view, extracellular recording of dorsal PAG neurons in freely behaving rats revealed a stronger response to innate than contextual predatory threats. Interestingly, close to 70% of the cells that had increased firing rate during the predatory context were also responsive to the innate threat (Bindi et al., 2022), suggesting the existence of a significant population of cells responsive to both innate and contextual threats (Reis et al., 2021b). Moreover, during innate and contextual predatory threats, an increase in cell firing was related to risk assessment responses in 49% of the dorsal PAG recorded cells (Bindi et al., 2022). In line with this view, *N*-methyl-D-aspartate (NMDA) receptor blockade or administration of a 5-HT1 agonist in the dorsal PAG has been shown to reduce risk assessment behaviors (Pobbe et al., 2011; Souza and Carobrez, 2016). These results indicate that the dorsal PAG should be related to the threat encoding and risk assessment during the predatory contextual fear.

As it stands, depending on the degree of the dorsal PAG activation, we may predict the behavioral outcome, where stronger activation observed during predator exposure is compatible with flight/freezing responses, and weaker mobilization observed in situations of higher ambiguity is compatible with risk assessment (Cezario et al., 2008). Notably, in a naturalistic threatening context, photoinhibition of the neural projections from the ACA to the dorsal PAG sharply decreased risk assessment and contextual avoidance during exposure to the predatory context (de Lima et al., 2022). In line with this result, it was demonstrated that mPFC neurons can specifically route sensory information to dorsal PAG by promoting aversion, and representing a potential circuit mechanism for valence processing and conflicting motivational outputs (Vander Weele et al., 2018). Taken together, the findings suggest the mPFC-dorsal PAG may dynamically influence dorsal PAG activity during conditioned threat processing and risk assessment behaviors, by impacting approach and avoidance states.

In a different threatening situation, the dorsal PAG is also involved in the expression of learned defensive responses elicited by exposure to a cue or a context previously associated with social defeat (Faturi et al., 2014). As in other learned fear conditions, animals defeated by conspecifics avoid the environment where the social defeat had occurred, and display a robust contextual fear response (Faturi et al., 2014). This response is characterized by a great deal of risk assessment and contextual avoidance. In line with this view, pharmacological blockade of the PMd and particularly of the dorsomedial part of the PAG reduced fear responses to a social defeat-associated context, decreasing risk assessment and contextual avoidance of the environment where the social defeat had occurred (Faturi et al., 2014). Animals exposed to a social defeat-associated context engage a neural pathway including the medial amygdalar nucleus along with the conspecific-responsive circuit in the medial zone of the hypothalamus, comprising the medial preoptic nucleus, ventrolateral part of the ventromedial nucleus, and the ventral premammillary nucleus, likely to process cues related to social memory (Faturi et al., 2014). In addition, social-defeated animals also mobilize a parallel circuit including the ventral subiculum and the juxtadorsomedial lateral hypothalamic area (Faturi et al., 2014). Interestingly, this parallel circuit is also recruited during conditioned avoidance processing observed in animals that have the choice to access a threatening zone

previously paired with footshocks (Viellard et al., 2016). Both paths merge into the PMd and allied PAG sites to organize defensive responses. As we have previously pointed out, risk assessment is a very complex response, and it is reasonable to believe that this elaborated behavioral display influenced by the dorsal PAG may be influenced by ascending projections to prosencephalic circuits. At this point, we can offer a speculative view of the circuits likely to mediate such responses.

As shown in Fig. 3, one of the main ascending targets of the dorsal PAG is the subfornical region of the lateral hypothalamic area (LHAsf) (Kincheski et al., 2012). Notably, the LHAsf provides a massive projection to the medial septal nucleus (Goto et al., 2005), which is known to provide cholinergic and GABAergic hippocampal inputs modulating theta oscillations (Vandecasteele et al., 2014). Hippocampal theta activity is likely to reflect states of fear (Sainsbury et al., 1987). In line with this view, different anxiolytics drugs have the common effect of reducing hippocampal theta frequency in a rather specific manner (Wells et al., 2013). Considering the hippocampus and its potential role in risk assessment responses, we call particular attention to the work of Blanchard's group, reporting that lesions in the ventral but not the dorsal hippocampus blocked risk assessment responses to different kinds of predatory threats (Pentkowski et al., 2006).

Particularly relevant for the setting of risk assessment is the strong link between the ventral hippocampus and mPFC, either through direct projections or indirect links involving amygdalar nuclei and nucleus accumbens (see Fig. 1). In fact, electrophysiological studies have shown that the correlation of theta frequency activity in the mPFC and ventral hippocampus was increased in anxiogenic environments (Adhikari et al., 2010). In the mPFC, single units with anxiety-related firing patterns are preferentially influenced by ventral hippocampal activity (Adhikari et al., 2011), thus supporting the idea that the hippocampal influence on the mPFC is critical to modulating anxiety. This is supported by multiple studies that have shown that the mPFC plays a role in anxiety tests that require the hippocampus (Lacroix et al., 2000; Shah and Treit, 2004).

Moreover, it is noteworthy that the dorsal PAG may also project to the intralaminar thalamic nuclei (i.e., central lateral and central medial thalamic nuclei) (Kincheski et al., 2012), which may represent another route to influence cortical networks (Furlong et al., 2010). Importantly, these are only speculative views on how the dorsal PAG would be related to prosencephalic targets organizing risk assessment responses, and further studies are needed to address this matter (Motta et al., 2017).

4. PAG and acquisition of fear memory

The dorsal PAG is involved in memory acquisition of shock-based fear conditioning (Yeh et al., 2021) and contextual fear memory to predator threats (de Andrade Rufino et al., 2019; Souza and Carobrez, 2016). In line with this view, several studies using classical fear conditioning to sound-, light-, or odor-CS showed that electrical, chemical, or optogenetic stimulation of the dorsal PAG support associative learning (Deng et al., 2016; Di Scala et al., 1987; Di Scala and Sandner, 1989; Kim et al., 2013; Kincheski et al., 2012). Interestingly, intra -dorsal PAG *N*-Methyl-D-Aspartate (NMDA) infusion during olfactory fear conditioning showed that immediate defensive responses rely on NMDA receptors, and aversive learning depends on the fine-tuning of metabotropic glutamate and AMPA receptors located in pre- and postsynaptic membranes (Back and Carobrez, 2018). Conversely, pharmacological or optogenetic inactivation of the dorsal PAG impaired the acquisition of fear memories during shock-based fear conditioning (Yeh et al., 2021) and exposure to predatory threats (de Andrade Rufino et al., 2019). Therefore, the dorsal PAG may be viewed as an important site to influence fear learning. In contrast, Silva et al. (2016) showed that dorsal PAG pharmacogenetic inactivation in mice during exposure to a predator (i.e., a rat) did not influence the acquisition of contextual fear memory. These discrepancies indicate that experimental protocol differences may influence the involvement of the PAG in fear learning.

As shown in Fig. 4, the dorsal PAG, particularly its dorsolateral part, provides massive ascending projections to the anterior hypothalamic nucleus and the subfornical region of the lateral hypothalamic area, both of which are known to provide strong inputs into the PMd; (Goto et al., 2005; Risold et al., 1994), where marked activation has been reported in response to electrical stimulation aimed at the dorsal PAG (Vianna et al., 2003). The PMd appears as a critical element in the ascending pathway involved in fear learning. Thus, using dorsal PAG activation as US, PMd beta-adrenergic blockade has been shown to impair fear conditioning in a paradigm that associates neutral odor cues as the CS and chemical stimulation of dorsal PAG as the US (Kincheski et al., 2012). In fact, the PMd seems to work as a critical hub to transfer information to the cortico-hippocampal-amygdalar circuits involved in processing fear memories through its main thalamic target, i.e., the ventral part of the anteromedial thalamic nucleus (Canteras and Swanson, 1992). Notably, a significant reduction in contextual fear responses was found after photoinhibition of the projection from the anteromedial thalamic nucleus to the ACA during cat exposure, where the ACA is thought to provide predictive relationships between the context and the threatening stimuli, and to influence memory acquisition through its projection to the basolateral amygdala and perirhinal region (de Lima et al., 2022).

In addition to the hypothalamus, the dorsal PAG provides several parallel thalamic paths likely to influence fear learning. Of particular interest, the dorsal PAG provides direct inputs to the paraventricular thalamic nucleus, the suprageniculate nucleus, and the central lateral nucleus (Kincheski et al., 2012; Yeh et al., 2021). Optogenetic silencing of the pathway from the dorsolateral PAG to the anterior paraventricular thalamic nucleus (aPVT) reduced aversive learning in a shock-based fear conditioning, an effect likely mediated by specific aPVT cell types that project to the lateral amygdalar nucleus (Yeh et al., 2021). It is

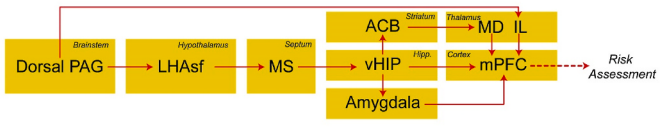

Fig. 3. Putative circuits involved in the dorsal PAG mediation of risk assessment responses. See text for discussion. Abbreviations: ACB – nucleus accumbens; Dorsal PAG – periaqueductal gray, dorsal part; IL – intralaminar thalamic nuclei; LHAsf – lateral hypothalamic area, subfornical region; MD – mediodorsal thalamic nucleus; mPFC – medial prefrontal cortex; MS – medial septal nucleus; vHIP – ventral hippocampus.

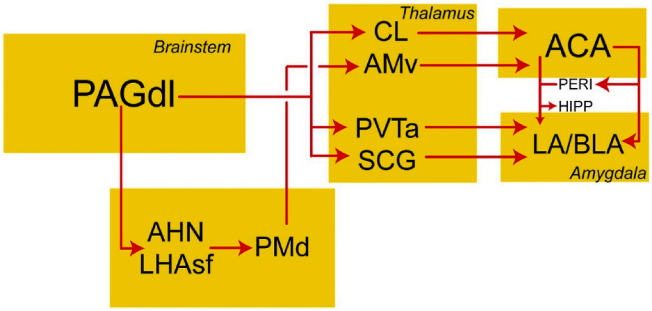

Fig. 4. Ascending projections from the dorsolateral PAG to hypothalamic and thalamic targets likely to influence fear memory acquisition. See text for discussion. Abbreviations: ACA – anterior cingulate area; AHN – anterior hypothalamic nucleus; AMv – anteromedial thalamic nucleus, ventral part; BLA – Basolateral amygdalar nucleus; CL – central lateral thalamic nucleus; HIPP – hippocampal formation; LA – lateral amygdalar nucleus; LHAsf – lateral hypothalamic area, subfornical region; PAGdl – periaqueductal gray, dorsolateral part; PERI – perirhinal area; PMd – dorsal premammillary nucleus; PVTa – paraventricular thalamic nucleus, anterior part; SGN – suprageniculate nucleus.

noteworthy that other thalamic dorsal PAG targets, particularly the suprageniculate nucleus, also project densely to the lateral amygdalar nucleus (Linke et al., 2000) and is a potential candidate to mediate fear learning. The central lateral nucleus, similarly to the anteromedial thalamic nucleus, also contained a large population of ACA projecting cells active during cat exposure (de Lima et al., 2022) and, therefore, may represent a putative site to influence the acquisition of fear memory in response to predatory threats. In support of the role of the dorsal PAG in influencing the lateral amygdala in fear learning, it was shown that basolateral amygdala inactivation disrupted fear conditioning using dorsal PAG stimulation as the US (Deng et al., 2016; Di Scala et al., 1987; Di Scala and Sandner, 1989; Kim et al., 2013; Kincheski et al., 2012). In contrast to the dorsal PAG, stimulation of the ventrolateral PAG does not support fear learning (Deng et al., 2016; Di Scala et al., 1987; Di Scala and Sandner, 1989; Kim et al., 2013; Kincheski et al., 2012). Moreover, cytotoxic lesion in the ventrolateral PAG did not disrupt learned contextual fear responses to cat exposure (de Andrade Rufino et al., 2019), and optogenetic inactivation of the ventrolateral PAG did not influence the acquisition of aversive memory during shock-based fear conditioning (Yeh et al., 2021). In fact, it has been shown that the ventrolateral PAG plays a role in instructing associative plasticity during fear conditioning. Aversive US might act as teaching signals, and the strength of this teaching signal is modulated by the expectation of the US. It is well known that Pavlovian association formation depends on prediction error – a discrepancy between the predicted outcome of the conditioning trial and the actual outcome of that trial. This error determines whether the US is effective in supporting learning or not, so that unexpected USs are more effective in supporting learning than expected US (McNally et al., 2011; Li and McNally, 2014). Aversive USs might act as teaching signals, and the strength of this teaching signal is modulated by the expectation of the US. The ventrolateral PAG is part of the nociceptive pathway, and US-evoked responses therein decreased as the shock became expected (Johansen et al., 2010). Information about the expectation of an aversive US to the ventrolateral PAG is mediated by a descending path from the medial part of the central amygdalar nucleus and involves ventrolateral PAG cells expressing μ-opioid receptors (McNally et al., 2011). Conversely, ascending paths from the ventrolateral PAG conveying teaching signals to instruct lateral amygdala associative plasticity involve the mid-line and intralaminar thalamic nuclei and the PFC, all of which are known to respond preferentially to unexpected–aversive US pairings (for review see (Li and McNally, 2014; McNally et al., 2011).

As it stands, the dorsal PAG provides instructive signals to prosencephalic regions in response to aversive events to produce fear learning, while the ventrolateral PAG is activated by cues predicting noxious stimulus to set memory strength proportional to the intensity of the aversive experience.

5. Human studies on PAG activation patterns during threat exposure

The first work on the human PAG, performed in the 1960's by Nashold and colleagues (Nashold et al., 1969) showed that stimulation of the PAG resulted in the intense emotions of fear and anxiety, including feelings of terror, fear of death, and which lead to a reluctance to continue stimulation. These subjective experiences are accompanied with changes in systolic and diastolic blood pressure, tachycardia and changes in respiration (Faull and Pattinson, 2017). Several studies have attempted to investigate the role of the PAG in defensive responses and more generally, negative affect. One of the first fMRI studies to investigate defensive responses in the PAG showed that as subjects were under attack from a looming virtual predator with the capacity to chase, capture and shock, there was a shift from the vmPFC to the PAG as the virtual predator moved closer to the subject (Mobbs et al., 2007). This result was replicated (Mobbs et al., 2009), and was extended on by showing that as a tarantula was placed closer to a subject's foot, there was an increase in midbrain activity (Mobbs et al., 2010). Others have used high-resolution imaging (i.e., 7 T) to support the role of negative affect in the human PAG (Satpute et al., 2015). Interestingly, the human fMRI has shown that the PAG is involved in pain-related learning, where it may receive value-related input from the vmPFC and the putamen (Roy et al., 2014). In turn, the PAG may send prediction error signals to a number of medial prefrontal areas including the OFC and ACA (Roy et al., 2014). This suggests that the PAG is not just a motor output region, but an active modulator of defensive decisions and learning.

The first study of escape in humans used a modified version of the flight initiation distance task, where one measures the distance at which the subject will escape from an approaching threat (Ydenberg and Dill, 1986). Qi and colleagues showed that either a fast or slow attacking threat that can cause pain upon capture of their avatar, only the fast escape decisions resulted in PAG activity. For slow escape decisions, the hippocampus and vmPFC were recruited (Qi et al., 2018). In a follow-up study, early escapes to slow, but not fast, attacking threats, were associated with trait anxiety and both the hippocampus and vmPFC increased in activity with individual differences in trait anxiety (Fung et al., 2019). This suggests that the PAG is not associated with trait anxiety and may be involved in more reflexive escape (Mobbs et al., 2020).

It is not yet known if the PAG is involved in freezing in humans. Indeed, while different types of freezing have been tied to the PAG (Brandão et al., 2008; Carvalho et al., 2018; 2013), research with human subjects has yet to directly show this region's role in freezing. The main obstacles are experimental design and the ethical considerations concerning how much fear is needed to induce freezing. However, Hashemi and colleagues (Hashemi et al., 2019) created a clever experiment using virtual reality. While subjects were being scanned using fMRI, participants played a shooting game where they had to shoot an avatar who would draw a phone or a gun. If they were too slow to shoot the avatar drawing the gun, they would receive an electric shock. Interestingly, the PAG was linked to preparation for action, which has been shown to result in freezing. Indeed, other markers of freezing (i.e., bradycardia) have been shown in the PAG using fMRI (Hermans et al., 2013). While these are indirect markers, they support the role of the human PAG in freezing related responses.

Despite little empirical research on freezing in humans, recent neuroimaging studies in humans have indicated that similar brain regions may be involved in human freezing. The fundamental nature of tonic immobility in anxiety responses has been investigated in humans in the context of a biological challenge. Human subjects reported either perceptions of immobility or a significant desire to flee during CO2 inhalation (Schmidt et al., 2008). The subjective experiences during either CO_2 challenge or spatial threat avoidance paradigm (Mobbs et al., 2007) can be associated with the fear responses triggered on the predatory imminence spectrum.

Previous studies pointed to similarities between the neural mechanisms involved in freezing and the shift to fight-or-flight action across humans and rodents. For example, flexibly shifting between freezing and active defensive modes is critical for adequate stress-coping and relies on fronto-amygdala connections (Likhtik et al., 2014; Roelofs, 2017) and frontal-midbrain neural dynamics (Mobbs et al., 2007; Reis et al., 2021a; Rozeske et al., 2018).

Overall, these data indicate that, in both humans and rodents, dynamic changes on threat imminence experience are encoded in PAG activity. Both preparation and behavioral action are functions encoded by the PAG that ultimately impact adaptation and survival. This evidence suggests that PAG activity can encode internal brain states related to the ongoing or anticipation of aversive events.

6. Conclusion

The studies reviewed above indicate that, in addition to the classical role of PAG as a brain region mainly involved as an output for defensive

responses, complex behavioral states can also be regulated and encoded in the PAG.

Due to the PAG's cellular organization and complex connectivity with wide brain circuits that coordinate multivariate motivational states, the dynamics of several neurotransmitter systems can play a fine-tuning regulatory role in the emergence of behavioral states. Animal and clinical data indicate that the PAG influences behavioral states through dynamic top-down and bottom-up forebrain connections (Mobbs et al., 2010; Motta et al., 2017; Silva et al., 2022; Silva and McNaughton, 2019). Studies investigating the functionality and connectivity of genetically identified cell populations of the PAG may reveal novel neurobiological mechanisms and new conceptual behavioral organization models of defensive responses and emotional states. Future studies focusing on dissecting the function of the PAG's afferent and efferent projections are also expected to provide new insights into this region's function.

Lastly, although the results discussed here have mostly focused on fear and defensive responses, based on the descriptions of the PAG's role in other survival functions, it is reasonable that the investigation of the interaction between defensive systems and other survival circuits are necessary to understand the broader and complex role of PAG on the adaptation and organism's survival.

Author contributions

FMCVR and AA conceptualized the review. AA and FMCVR wrote the abstract and introduction. FMCVR wrote the innate fear section and conclusion, NSC wrote the learned fear section, and DM wrote the human fear section.

Declaration of competing interest

None of the authors has relevant financial interests to disclose or a conflict of interest.

Data availability

No data was used for the research described in the article.

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