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Research report

Effects of alprazolam and cannabinoid-related compounds in an animal model of panic attack

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HIGHLIGHTS

- The pharmacological treatment of panic disorder is limited.
- One of the few animal models of panic is the response to KCN injection in rats.
- The KCN model is useful for screening new potential treatments for panic disorder.
- Cannabinoid compounds, which may alleviate anxiety, failed to inhibit KCN effects.
- Alprazolam, a benzodiazepine, abolished KCN behavioral and cardiovascular effects.

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ABSTRACT

Selective stimulation of carotid chemoreceptors by intravenous infusion of low doses of potassium cyanide (KCN) produces short-lasting escape responses that have been proposed as a model of panic attack. In turn, preclinical studies suggest that facilitation of the endocannabinoid system attenuate panic-like responses. Here, we compared the effects of cannabinoid-related compounds to those of alprazolam, a clinically effective panicolytic, on the duration of the escape reaction induced by intravenous infusion of KCN (80 μg) in rats. Alprazolam (1, 2, 4 mg/kg) decreased escape duration at doses that did not alter basal locomotor activity. URB597 (0.1, 0.3, 1 mg/kg; inhibitor of anandamide hydrolysis), WIN55,212-2 (0.1, 0.3, 1 mg/kg; synthetic cannabinoid), arachidonoyl-serotonin (1, 2.5, 5 mg/kg; dual TRPV1 and anandamide hydrolysis inhibitor), and cannabidiol (5, 10, 20, 40 mg/kg; a phytocannabinoid) did not decrease escape duration. Alprazolam also prevented the increase in arterial pressure evoked by KCN, while bradycardia was unchanged. This study reinforces the validity of the KCN-evoked escape as a model of panic attack. However, it does not support a role for the endocannabinoid system in this behavioral response. These results might have implications for the screening of novel treatments for panic disorder.

1. Introduction

Panic disorder is a subtype of anxiety disorder characterized by the occurrence of panic attacks, which comprises feelings of fear, discomfort and somatic symptoms of respiratory distress, hyperventilation, palpitations, and sweating [1]. These attacks can be classified as either 'respiratory' or 'non-respiratory', depending on its symptoms [2,3]. Some attempts to explain their neural substrates have been focused on brainstem structures such as the locus coeruleus, the nucleus raphe magnus, and the periaqueductal gray matter (PAG) [4–6]. Other theories emphasized prosencephalic structures such as the hypothalamus, the amygdala, and the prefrontal cortex [7–11].

Among the main theories regarding the neurobiology of panic attacks, Klein's (1993) suffocation false alarm (SFA) theory states that clinical panic attacks are the misfiring of a suffocation alarm system. The false activation of the suffocation alarm would lead to dyspnea, fear, and hyperventilation [12,13]. The neural substrate of the 'suffocation alarm system', however, has remained elusive. In particular, the participation of the locus coeruleus in panic attacks was contradicted by the lack of aversiveness of its stimulation in humans [4]. The involvement of the amygdala was questioned by a study showing that patients with bilateral damage to the amygdala develop panic attacks both spontaneously and in response to the

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inhalation of 35% CO2 and infusion of isoproterenol [14–16]. As to cognitive theories, it is proposed that the panic attack is the cortical 'catastrophization' of bodily symptoms [11]. Although all theories suggest that panic attacks are false alarms, the neural mechanisms that either predispose or trigger the response remain unknown.

In this context, recent data suggest that the PAG harbors a hypoxia-sensitive suffocation alarm system. Activation of this structure is hypothesized to trigger a respiratory-type panic attack and render the subject hypersensitive to CO2 [17,18] Evidence for that were obtained from studies utilizing the escape reaction induced by potassium cyanide (KCN) as an animal model of panic attack. These studies showed that low doses of KCN induce an escape reaction that is blocked by clinically effective panicolytic drugs and electrolytic lesions of the DPAG [18,19]. Moreover, it was shown that the escape response was potentiated by hypercapnia [18]. Intravenous infusion of KCN leads to activation of peripheral chemoreceptors resulting in hypertension, bradycardia and tachypnea, which constitute the chemoreflex [20], along with a panic-like increase in locomotor activity.

Studies employing other animal models suggest a possible involvement of the endocannabinoid system in panic-like responses [21–25]. This system comprises the CB_1 and CB_2 receptors, the endogenous ligands anandamide and 2-arachidonoyl glycerol (2-AG), and the enzymes mediating their synthesis and breakdown [26]. Activation of CB_1 receptors in the PAG attenuates the escape reaction induced by stimulation of this structure [21–24,27,28]. CB_1 agonists injected either systemically or in the PAG also attenuate the escape reaction induced by 20 kHz ultrasound and exposure to the elevated T maze [24,25,28]. Moreover, compounds that inhibit fatty acid amide hydrolyse (FAAH), the main enzyme responsible for anandamide hydrolysis, also induce panicolytic-like effects [23,25]. Finally, these effects also occur after blockade of the transient receptor potential vanilloid type-1 (TRPV1) channel, which is also a molecular target for anandamide [26]. Based on these models, CB_1-mediated signaling might have a role in counteracting aversive reactions [29]. However, other models based on different theories of panic disorder could unveil a different role for the system in panic-like responses.

Accordingly, in this study we compared the effects of alprazolam, a benzodiazepine with therapeutic use for the treatment of panic disorder, to those of cannabinoid-related compounds on the panic-like reaction induced by intravenous infusion of a low dose of KCN. We tested the anandamide hydrolysis inhibitor, URB597; the dual FAAH/TRPV1 blocker, arachidonoyl-serotonin; the non-selective cannabinoid agonist, WIN55,212-2; and cannabidiol (CBD), a phytocannabinoid that might act as a 5-HT_{1A} receptor agonist [30] and presents anxiolytic (humans and rodents) and panicolytic (rodents) effects [31,32].

2. Materials and methods

2.1. Animals

Male Wistar rats (250–350 g) from the animal facility of the Institute of Biological Sciences of UFMG were used. Animals were kept in a room with controlled temperature (24 °C), 12-h light–dark cycle, and free access to food and water. They were housed in groups of five per cage. All procedures were approved by the Committee for Ethics in Animal Experimentation (protocol 259/2013).

2.2. Drugs

All drugs were injected via ip route, except for KCN, which was injected via iv route. KCN (Merck, Darmstad, Germany) was diluted in saline and the dose (80 μg/0.1 mL) was chosen based on a dose-response curve performed in our laboratory (data not shown) and previous reports [19]. Alprazolam (1, 2, and 4 mg/kg; EMS Brazil) was dissolved in a solution of saline and 2% Tween. WIN55,212-2, (0.1, 0.3, and 1 mg/kg; Cayman Chemicals), URB597 (0.1, 0.3, and 1 mg/kg; Cayman Chemicals), and arachidonoyl-serotonin (1, 2.5, and 5 mg/kg; Cayman Chemicals) were dissolved in a solution of ethanol, cremophor and saline in a proportion of 1:1:18. CBD (5, 10, 20, and 40 mg/kg) was dissolved in a solution of Tween 5% and saline. Doses were chosen based on the following studies: [25,33,34].

2.3. Femoral artery and vein catheterization

One day before the experiments, under tribromoethanol (250 mg kg^{-1} I.P.) anaesthesia, a small incision was made in the inguinal region to expose the femoral artery and vein. Polyethylene catheters (PE-10 connected to PE-50; ClayAdams, Parsippany, NJ,USA) filled with saline (NaCl 0.9%) were inserted into the abdominal aorta via the femoral artery and into the femoral vein. Both catheters were tunnelled subcutaneously and exteriorized through the back of the neck. The catheter inserted in the femoral artery was used for recording of cardiovascular parameters and the catheter in the femoral vein was used for intravenous infusion. After surgeries, rats were placed in individual cages.

2.4. Chemoreflex stimulation

The peripheral chemoreflex was activated by intravenous infusion of 100 μL of KCN (80 μg/0.1 mL) in accordance with the procedures described by [20] and previously validated for our experimental conditions [35,36].

2.5. Recording of cardiovascular parameters

The catheter in the femoral artery was flushed with heparinized saline (0.9% NaCl) to prevent clotting and then connected to the pressure transducer (model CDX III; Cobe Laboratories, Lakewood, CO, USA). Pulsatile arterial pressure (PAP) was continuously recorded by an A/D data acquisition system (MP100; Biopac Systems, Inc., Santa Barbara, CA, USA). Mean arterial pressure (MAP) and heart rate (HR) was simultaneously derived from arterial pulse waves by software (AcqKnowledge 5; Biopac Systems).

2.6. Behavioral measurement

For measuring escape duration, animals were placed in a rectangular box (50 cm long, 30 cm wide, 20 cm high) with opaque plastic walls. Animals were left in the box for 10 min for habituation. Afterwards, KCN was injected and the behavior response was recorded by a videocamera situated on top of the box. Escape duration was measured manually by an experimenter blind to the treatments.

2.7. Experimental protocols

For the behavioral experiments, animals received ip injections of cannabinoids and related drugs that act on the endocannabinoid system (WIN55,212-2, URB597, AA-5-HT, and CBD) or alprazolam. Twenty minutes after the first injection they were placed in the box, left undisturbed during 10 min for habituation and received KCN infusion. The distance traveled was measured after the beginning of KCN infusion. In an independent experiment, for measurement of basal locomotor activity, the same protocol was followed, except that no KCN was administered. The distance traveled in the box was measured with the aid of the software Any-Maze, version 4.99 (Stoelting). For the cardiovascular recording, animals received one

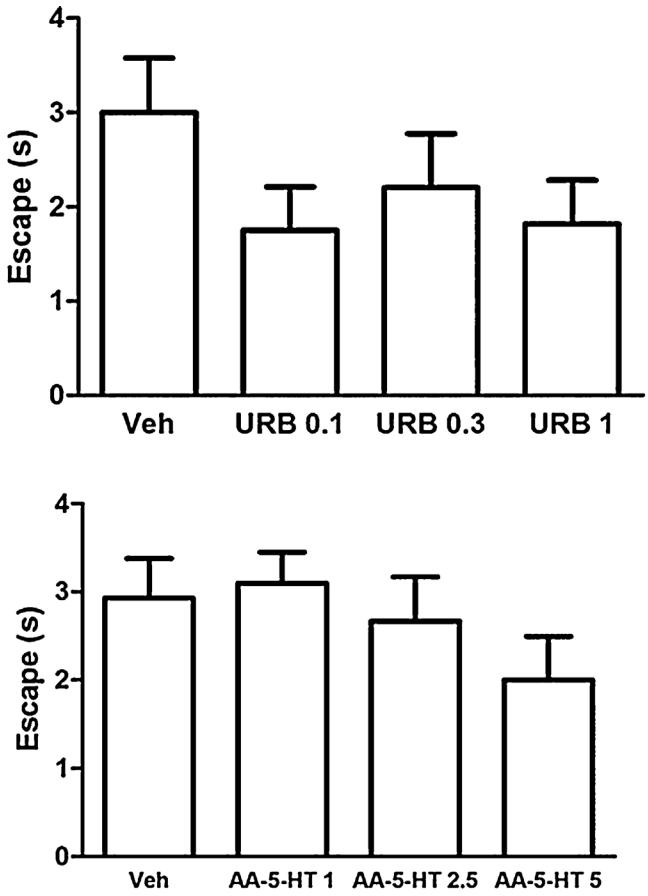

Fig. 1. Upper panel: Effects of ip injections of URB597 (URB, 0.1, 0.3 and 1 mg/kg) or vehicle (Veh) on the duration of KCN-induced escape reaction in rats; no statistical difference was revealed by ANOVA followed by Newman Keuls' test. p = 0.31, n = 11, 12, 10, 11. Lower panel: Effects of ip injections of arachidonoyl-serotonin (AA-5-HT 1, 2.5 and 5 mg/kg) or vehicle (Veh) on the duration of KCN-induced escape reaction in rats; no statistical difference was revealed by ANOVA followed by Newman Keuls *post-hoc* test. p = 0.37; n = 14, 10, 9, 10.

infusion of KCN (KCN 1) and the heart rate and mean arterial pressure were recorded. Then, animals received alprazolam or vehicle and after 30 min, they received another infusion of KCN (KCN 2). This protocol was chosen because some animals present an abnormal hypotensive response to the infusion of KCN, hence it was necessary to exclude them from the experiment. This protocol is validated and routinely performed for the study of the physiology of the chemoreflex [35].

2.8. Statistical analysis

Data from the behavioral experiments were analyzed by one-way ANOVA and Newman Keuls as a *post-hoc* test. Data from the cardiovascular recordings were analyzed by two-way ANOVA with repeated measures. *Post-hoc* analyses were performed with the Bonferroni test. All data are expressed as mean and SEM. Statistical significance was considered when $p < 0.05$.

3. Results

All the cannabinoid-related compounds failed to attenuate KCN-induced panic-like responses. The statistical analyses revealed the they have the following effects in the duration of the escape reaction: URB597 (inhibitor of anandamide hydrolysis), $F(3,39) = 1.21$, $p = 0.31$ (Fig. 1, upper pannel); AA-5-HT (dual FAAH/TRPV1 blocker), $F(3,39) = 1.07$, $p = 0.37$ (Fig. 1 lower pannel); WIN,55-212-2 (cannabinoid non-selective agonist), $F(3,37) = 1.45$, $p = 0.24$ (Fig. 2, upper panel); and CBD (multiple mechanisms), $F(4,48) = 2.23$; $p = 0.07$ (Fig. 2, lower panel). Contrary to the above results, alprazolam decreased escape duration at all doses tested, $F(3,38) = 5.30$, $p = 0.003$ (Fig. 3, upper panel). This compound did not reduce basal locomotion, $F(3,20) = 2.62$, $p = 0.07$ (Fig. 3, lower panel). Moreover, alprazolam decreased the pressor response induced by KCN: interaction between factors $F(1,12 = 5.27)$, $p = 0.04$; alprazolam effect $F(1,12) = 9.17$, $p = 0.01$; trial effect $F(1,12) = 8.84$, $p = 0.01$ (Fig. 4, upper panel); this drug, however, did not alter the bradycardia: interaction between factors $F(1,12) = 5.78$, $p = 0.03$; alprazolam effect $F(1,12) = 4.68$, $p = 0.4$; trial effect $F(1,12) = 0.04$, $p = 0.83$ (Fig. 4, lower panel).

4. Discussion

The present study showed that alprazolam, a benzodiazepine clinically effective for the treatment of panic disorder, prevented KCN-induced escape and pressor responses. These effects corroborate previous results showing that KCN-evoked escape is abolished by clinically-effective doses of clonazepam and chronic treatment with the selective serotonin reuptake inhibitor fluoxetine [19]. In the same vein, alprazolam and fluoxetine attenuates escape to severe hypoxia (8% O_2) [37]. In addition, the hypoxia-evoked escape was attenuated by a 5-HT_{1A} receptor agonist injected in the DPAG [37]. Taken together, these data suggest that the panic-like behavior can be modulated by GABAergic and serotonergic receptors at the PAG. KCN-evoked pressor response is also attenuated by alprazolam. The latter effect is in line with alprazolam attenuation of both pressor and tachycardic responses to lactate infusion in "panic-prone" rats [38].

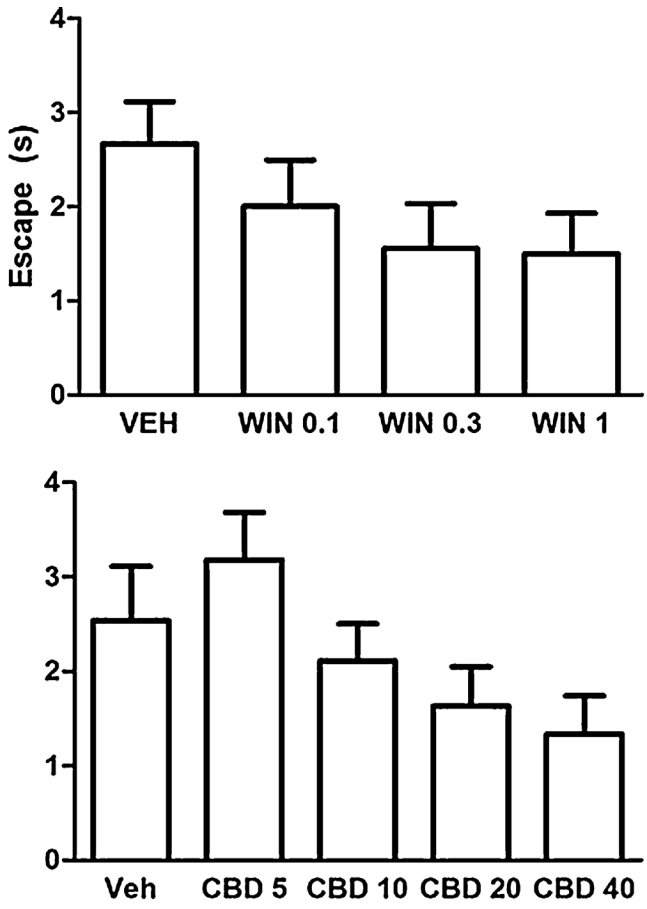

Fig. 2. Upper panel: Effects of ip injections of WIN55-212,2 (WIN 0.1, 0.3 and 1 mg/kg) or vehicle (Veh) on the duration of KCN-induced escape reaction in rats; no statistical difference was revealed by ANOVA followed by Newman Keuls *post-hoc* test. p = 0.24, n = 12, 10, 9, 10. Lower panel: Effects of ip injections of cannabidiol (CBD, 5, 10, 20 and 40 mg/kg) or vehicle (Veh) on the duration of KCN-induced escape reaction in rats. no statistical difference was revealed by ANOVA followed by Newman Keuls *post-hoc* test. p = 0.07; n = 13, 11, 9, 11, 9.

On the other hand, alprazolam failed to inhibit KCN-evoked bradycardia. These results agree with the notion that the increase in arterial pressure and the bradycardia have different neural substrates. Accordingly, inactivation of the dorsomedial hypothalamus, a brain region involved in defensive responses, attenuates only the behavioral and pressor response to intravenous infusion of KCN [39]. Future studies should clarify the involvement of other regions related to defensive behavior in the KCN-induced escape reaction. Moreover, other aspects of emotional responses associated with anxiety and panic, such as activation of the HPA axis, warrants further investigation. Nonetheless, it was shown that intermittent activation of chemoreceptors by KCN does not induce c-Fos protein expression in the median eminence of the paraventricular nucleus of the hypothalamus [40]. Interestingly, this would be in agreement with the proposal that there is no activation of the hypothalamic-pituitary-adrenal axis in panic attacks and models of panic attacks [41].

Although the endocannabinoid system has been proposed as a protective mechanism against pain and fear [23,42–44], cannabinoid-related drug did not alter KCN-evoked escape in this study. We have tested if facilitation of anandamide signaling, by inhibition of its hydrolysis with URB597 (an FAAH inhibitor), would attenuate the escape reaction induced by KCN. URB597 may have therapeutic potential, since it is well tolerated in monkeys and exerts anxiolytic and panicolytic effects in rodent models of anxiety and panic [29,45,46]. In this study, however, we found no effect of URB597 on escape duration. Since TRPV1 channels also modulate panic-related responses we hypothesized that the dual FAAH/TRPV1 blocker, AA-5HT, would attenuate the aversive state generated by KCN [47]. Contrary to our expectations, we found no effect for this compound. One possible explanation for the lack of effects of these compounds is that the aversive stated generated by KCN might not promote enough synthesis of anandamide in order for it to activate CB1 receptors. On the contrary, high-intensity aversive stimuli, such as pain and electrical stimulation of the PAG, do promote anandamide release in this region [41–43]. Based on this assumption, we tested if a compound that directly target cannabinoid receptors, WIN,55-212-2, would be effective in this model. This compound, however, also failed to decrease escape duration significantly, discarding a prominent role for CB_1 receptors in this response. Finally, although CBD was previously shown to exert a panicolytic effect via 5-HT1A activation, it did not decrease escape duration [30,34]. The lack of effects of the drugs tested is in contrast to an extensive literature showing that these drugs exert anti-aversive effects [31,47].

An important characteristic of an animal model of panic attack is the capability of the model to detect new panicolytic agents with different mechanisms of actions [17,48,49]. Otherwise, the animal model would be limited to a few classes of drugs that are already in clinical practice. Considering this, the escape reaction induced by KCN might not represent a reliable measure to detect panicolytic agents that facilitate anandamide and CB_1 signaling. Another interpretation for our results is that these drugs may not be promising candidates for the treatment of panic attacks. It should be noted

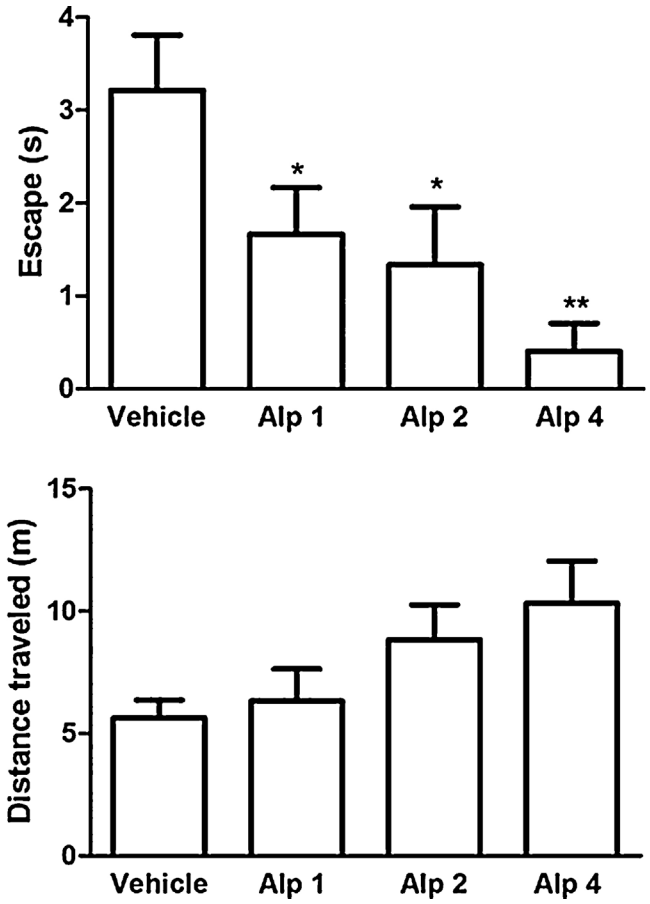

Fig. 3. Upper panel: Effects of ip injections of alprazolam (Alp, 1, 2 and 4 mg/kg) or vehicle (Veh) on the duration of KCN-induced escape reaction in rats; alprazolam reduced the effect of KCN, as revealed by the Newman Keuls's test. *p < 0.05, **p < 0.01, n = 14, 9, 9, 10. Lower panel: Effects of ip injections of alprazolam (Alp, 1, 2 and 4 mg/kg) or vehicle (Veh) on locomotor activity in rats after habituation (10 min) to the arena; no statistical difference was revealed by ANOVA followed by Newman Keul's test. p = 0.07; n = 6/group.

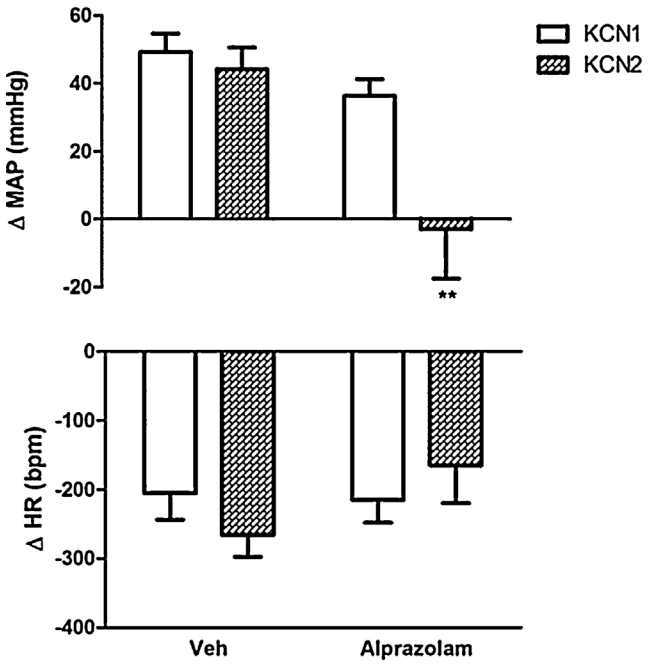

Fig. 4. Effects of KCN infusions before (KCN1) and after (KCN2) vehicle or alprazolam injections in rats. Upper panel: Alprazolam (4 mg/kg) abolished the hypertensive response to the second infusion of KCN (KCN2); Bonferroni's test, **p < 0.01, n = 7/group. Lower panel: Alprazolam (4 mg/kg) did not modify the bradycardic response to KCN; no effect was revealed by Two-Way ANOVA or Bonferroni's test. p = 0.4 for drug effect; n = 7/group.

that panicogenic and anxiogenic effects of cannabinoids have been reported. Δ-9-THC, a partial agonist at CB_1 receptors, may induce anxiogenic effects [50,51], which can also occur after high doses of synthetic cannabinoids [52,53]. Another possibility for future studies would be to investigate the effects of chronic treatment with cannabidiol and to clarify the role of other endocannabinoids, such as 2-AG, in the panic-like behavior evoked by KCN.

The majority of the studies investigating these cannabinoid-related drugs in models of panic attacks were performed with central injections directly targeting specific brain regions. However, the use of systemic injections may be more interesting for the study of a potential therapeutic effect. For this reason, the pharmacokinetics aspects of these compounds shall be considered. URB597 is rapidly distributed to serum and brain tissue and produces a complete blockade of brain FAAH approximately after 10–15 min of injection [53]. This characteristic makes it favorable for the study with systemic injections. However, higher doses may interact with esterases in liver which could limit the availability of the drug in the tissues [54]. As for the WIN-55,212-2, doses employed here accumulate in the plasma, brain and adipose tissue dose-dependently 30 min after injection which also allows for studies with systemic injections [55,56]. Pharmacokinetic properties of cannabidiol show that it has also a high bioavailability and a 24 h half-life [56]. Thus, although we did not measure plasma levels, previous studies and the pharmacokinetics aspects suggest that low dose or poor drug absorption and distribution do not account for the lack of effect in the KCN model of panic.

Finally, the response induced by KCN may be more relevant for the understanding of a specific subtype of panic disorder, namely the respiratory subtype. In these cases, patients present chest pain, fear of dying, dyspnea, and feeling of suffocation, whereas in the non-respiratory subtype these symptoms are absent [2,3]. Moreover, these subtypes seem to respond differently to pharmacotherapy [57]. For the respiratory subtype, patients seem to show a better improvement with chronic treatment with antidepressants, whereas for the non-respiratory subtype, alprazolam improved better and also had an acute therapeutic effect [56]. This suggests that, for the respiratory subtype, adaptations in the brain following chronic treatment may be important for the therapeutic effect to occur. In our study, cannabinoids drugs were injected acutely, similar to what has been done in previous studies with models of panic attacks and anxiety [29] Therefore, we can speculate that the potential therapeutic effect of cannabinoids drugs, which is observed in other models of panic attacks, might mimic the acute therapeutic effect of alprazolam.

Finally, there are studies investigating the role of central and peripheral chemoreflexes in panic attacks in humans [58–60]. One study found no difference in the threshold of the chemoreflex between normal volunteers and panic disorder patients [61]. As concluded by the study of Katzman et al., chemoreflexes may not be involved in the triggering of a panic attack, but may participate in the genesis of the attack.

In conclusion, this study shows that alprazolam reduces escape duration and prevents the pressor response induced by KCN in rats, further validating this animal model of panic attack. The fact that endocannabinoid-related compounds failed to modify this behavioral response may have implications for the understanding of the neurobiology of panic attacks and for the development of new treatments for panic disorder.

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