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Kindling epileptogenesis and panic-like behavior: their bidirectional connection and contribution to epilepsy-associated depression

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Abstract

Anxiety is one of the most common comorbidities of epilepsy, which has major detrimental effects on the quality of life. Generalized anxiety disorder (GAD) associated with epilepsy has been receiving most attention. However, several other forms of anxiety reportedly present in epilepsy patients, including panic disorder (PD). In this study, using an animal model of limbic epilepsy, we examined the interplay between epilepsy and panic-like behavior (PLB). Further, considering the high degree of comorbidity between depression on the one hand, and both epilepsy and PD on the other hand, we studied whether and how the presence of PLB in animals with epilepsy would affect their performance in depression-relevant tests. Fifty-day-old male Wistar rats were subjected to repeated alternating electrical stimulations of the basolateral amygdala (BLA) to induce kindling of limbic seizures, and the dorsal periaqueductal gray (DPAG) to induce panic-like episodes. Seizure susceptibility and panic reaction threshold were examined before the first and 24 hours after the last stimulation. At the end of the stimulations, the rats were examined in depression-relevant tests: saccharin preference test (SPT) for anhedonia and forced swimming test (FST) for despair/hopelessness. With regard to kindling, BLA+DPAG stimulation induced more profound increase of seizure susceptibility than BLA stimulation alone (evident as the reduction of the afterdischarge threshold and the increase of the afterdischarge duration). With regard to PLB, the BLA+DPAG stimulation exacerbated the severity of panic-like episodes, as compared with the DPAG stimulation alone. BLA stimulation alone had no effects on panic-like reactions, and DPAG stimulation alone did not modify kindling epileptogenesis. Combined stimulation of BLA and DPAG induced depressive-like behavioral impairments. This is the first experimental study

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Disclosure of Conflicts of Interest

The authors report no conflicts of interest.

showing bidirectional, mutually exacerbating effect of epilepsy and PLB, and the precipitation of depressive-like state by the epilepsy-PLB comorbidity.

Keywords

Epilepsy; Panic disorder; Depression; Comorbidity

1. Introduction

Anxiety disorders have been reported in 20% of people with epilepsy vs. 10% in people without epilepsy [1, 2]. In epilepsy patients, anxiety contributes to further deterioration of quality of life (QoL) [3, 4]. The anxiety disorder spectrum includes several types of disorders with different mechanisms and symptoms [5]. In the laboratory, the most commonly examined type is generalized anxiety disorder (GAD), while other types have received scarce if any attention. At the same time, epilepsy patients present with various types of anxiety. Particularly, panic disorder (PD) is one of the most common types, and may be even higher prevalence (5%) than GAD (3%) [1, 6]. PD is characterized by recurrent presence of panic attacks (PA), which are the hallmark of its diagnosis [7].

In rodents, phobic avoidance, a characteristic manifestation of the PD shares neural substrates that generate innate fear-induced reactions, such as freezing [8], running and jumping behaviors [9, 10]. Panic reactions are related to proximal threat and recruit brainstem structures [9], such as the dorsal raphe nucleus (DR) that contains organized subgroups of serotonergic neurons, which are involved in mechanisms of anxiety. Neurons in the dorsal region of DR (DRD) project into the forebrain and facilitate anxiety-related behaviors, including PA; while the neurons in the dorsal raphe ventrolateral (DRVL)/ ventrolateral periaqueductal gray (VLPAG) are involved in the inhibition of panic-reactions [11, 12]. Furthermore, the role of the dorsal periaqueductal gray (DPAG) in the expression of unconditioned defensive reactions and panic is well established [8, 13]. This process implicates ascending connections from DPAG to the prosencephalic centers, such as the amygdala, through the medial forebrain bundle, which allows the animal to assess the aversive situation and helps in the recognition of the frightening stimuli [8, 13]. In research setting, exogenous activation of the DPAG has been proposed as a rodent model of PD. Both chemical and electrical stimulation of DPAG produces panic-like behaviors (PLB) in rodents, similar to the responses exhibited by the animals in the presence of predators [8, 14]. In patients, the electrical stimulation of DPAG generates physiological changes that are similar to those observed during PA, such as autonomic alterations and unpleasant feelings [8, 15].

Aside from the PD-epilepsy connection, each of the two conditions has a high degree of comorbidity with depression [16–19]. Co-ocurrence of anxiety and depression in people with epilepsy has worse impact on their QoL than each of the disorders in isolation [3, 20]. In epilepsy patients with comorbid depression, the presence of PD, decreased the likelihood of remission of the depressive disorder [21].

Considering the discussed clinical aspects of PD-epilepsy-depression comorbidity, as well as the lack of experimental studies on this subject, we pursued to reproduce this comorbidity in the animal system, and to examine their interactions between seizures, panic-like and depressive-like impairments.

2. Material and methods

Experimental design is shown on Fig. 1A. The study was performed in a controlled randomized blinded fashion. Control animals were subjected to sham procedures. Randomization occurred during the assignment of the animals to various experimental and control groups. For the performance of all behavioral tests, the experimenter was unaware of the preceding procedures, to which the animals had been subjected. For the off-line data analysis, persons analyzing the data were blinded vis-à-vis all the procedures.

2.1. Animals

Male Wistar rats, 50 days old at the beginning of the study were maintained under controlled conditions (room temperature 20–26 °C, humidity 30–70%, 12 h light–dark cycles, food and water *ad libitum*). All experiments adhered to NIH regulations, and were approved by the UCLA Animal Research Committee.

2.2. Surgery

Under isoflurane anesthesia, rats were stereotaxically implanted with two bipolar stimulating electrodes (Plastics One, Roanoke, VA), one in the left basolateral amygdala (BLA, from Bregma: 2.5 mm posterior, 4.8 mm lateral, 8.5 mm ventral), and another in the DPAG (from Bregma: 7.3 mm posterior, midline, 4.5 mm ventral) [22]. A tripolar electrode for electroencephalographic (EEG) recordings was placed 3 mm anterior to Bregma with the ground connected to a screw on the nasal bone. The implantation sites were identified by histology using Nissl staining (Fig. 1B–C).

2.3. Electrical stimulations

Subjects were randomly assigned to one of four groups: control (BLA sham + DPAG sham, n=8); kindling+PLB (BLA stimulation + DPAG stimulation, n=8); kindling (BLA stimulation + DPAG sham, n=8), and PLB (BLA sham + DPAG stimulation, n=8). Kindling and PLB were induced in the same animals through intermittent stimulations of BLA and DPAG as described below (Fig. 1A).

2.4. Afterdischarge properties and kindling

As a model of epilepsy, we have chosen BLA kindling, as it affords graded limbic seizures, which are induced on demand [23, 24]. Seven days after surgery, the animals were connected to the DS8000 electrical stimulator via DSI100 stimulus isolators (World Precision Instruments, Sarasota, FL) and to the MP100/EEG100B acquisition system (BIOPAC, Santa Barbara, CA). The afterdischarge threshold (ADT), defined as a response of at least 2 seconds-long after the end of the stimulation (Fig. 2A), was detected first by applying trains of electrical stimuli (series of 10 seconds train duration, 20 Hz, 1 ms pulse

duration, square-wave monophasic stimuli, starting 0.1 mA, at 0.1 mA increments, applied every 10 minutes). Afterdischarge duration (ADD) was measured off-line.

Beginning from the two days after the afterdischarge detection, the animals were subjected to kindling, which consisted of three BLA stimulations a day, at 10:00, 14:00 and 18:00 h during seven consecutive days (Fig. 1A) using the parameters described above, starting with 50 μA above the ADT. Kindling state was confirmed by the presence of three consecutive stage 4–5 behavioral seizures [25] during the last day of the procedure. Twenty four hours after the last kindling trial, ADT and ADD were measured following the protocol described for their baseline detection.

2.5. Threshold detection and induction of PLB

One day after the determination of baseline afterdischarge properties, thresholds for PLB induced by DPAG stimulation, were measured. DPAG was stimulated with 30 s trains, 60 Hz, 1 ms, sine-wave pulses of stepwise increasing intensities, starting 0.1 mA, at 0.1 mA increments, applied 5 min apart. Threshold stimulations for eliciting PLB were detected. Behavioral reactions were quantified using the following ethogram [26]: *exophthalmus*, the eyes take on a spherical shape due to the eyeball protrusion and fully opening of the eyelid; *immobility*, general behavioral arrest accompanied by the increase in muscle tonus (extension of neck and/or limbs and elevation of head, trunk and/or tail during at least 3 s); *running*, fast locomotion and swing movements of anterior and posterior limb pairs; *jumping*, upward jumps directed to the edge of the cage. Beginning from the next day after baseline measurements, the animals underwent repeated DPAG stimulations, two stimulations a day, at 12:00 and 16:00 h (Fig. 1A), during seven consecutive days using the above-described parameters, starting with 50 μA above the threshold for inducing exophthalmus/immobility behavioral response. The threshold for each behavior was measured following the described procedure, one day after the last stimulation (30 minutes after measuring afterdischarge from BLA). Behaviors were video recorded and analyzed off-line in a blinded fashion.

2.6. Saccharin preference test

Saccharin preference test (SPT) was used to examine the presence of an anhedonia-like state. Two days after the last BLA+DPAG stimulation, two graduated bottles were introduced in the home cage, one filled with regular water, and the other with 0.1% saccharin solution. The bottles were left for 24 hours, after which the volume consumed from each bottle was recorded. Normal animals preferentially consume saccharin solution; “anhedonic” animals consume statistically similar volumes of saccharin and water [27, 28]. Taste preference was expressed as percent of the volume of saccharin solution of a total volume of fluid (saccharin plus water) consumed over 24 h [29].

2.7. Forced swimming test

The state of hopelessness/despair was examined using forced swim test (FST). One hour after the SPT, the animals were allowed to freely swim for 5 min in a plastic tank (60 cm height and 30 cm width), filled with water (3/4 of the tank) at 22–25 °C. Behavior was recorded by a top-mounted camera for off-line analysis. Two behavioral patterns were

analyzed: active swimming (swimming along and climbing on the walls and diving) and immobility (movements limited to maintaining head above water). Both behaviors are present in normal and depressed animals, but in models of depression, the animals spend longer time immobile, which is interpreted as a hopelessness/despair state. Cumulative time spent immobile was calculated [29, 30].

2.8. Statistical analysis

Data were analyzed using the Prism 6.0 software (GraphPad Software, San Diego, CA). Experimental groups that passed the D'Agostino-Pearson normality test were analyzed using one-way ANOVA, followed by Holm-Sidak's test. The experimental groups that showed different variances were analyzed with nonparametric Kruskal-Wallis one-way ANOVA, followed by Dunn's test. In all cases, data are presented as mean ± standard error of mean (SEM). A confidence level of 95% was accepted as significant.

3. Results

3.1. Effects of kindling and repeated stimulation of DPAG on the BLA excitability

All animals developed stage 4–5 seizures during the last day of kindling procedure. Twenty four hours after the completion of BLA and/or DPAG stimulations, the animals of both BLA-only and BLA+DPAG groups showed increased seizure susceptibility, which was evident as the decrease of ADT, and the increase of the ADD as compared with the baseline parameters (ADT, 0.82 ± 0.03 mA; ADD, 33.18 ± 0.98 s). In the animals of BLA+DPAG group, the excitability was significantly higher than in the animals of BLA-only group. DPAG stimulation alone neither had any effects on the BLA excitability (Fig. 2B), nor it induced behavioral or electrographic seizures, even at the maximum current to induce the most severe response (jumping) (Fig. 3A).

3.2. Effects of kindling and repeated stimulation of DPAG on PLB

Neither kindling alone, nor repeated stimulation of DPAG alone modified the threshold for the induction of PLB. At the same time, alternating repeated BLA and DPAG stimulations, led to significant decrease of threshold for the induction of all types of PLB as compared with the baseline values (exophthalmus, 0.36 ± 0.05 mA; running, 0.61 ± 0.06 mA; jumping, 0.73 ± 0.07 mA), except immobility that did not change when compared with the baseline result (0.36 ± 0.06 mA) (Fig. 3B–E). No PLB were observed after BLA kindling alone.

3.3. Effects of kindling and repeated stimulation of DPAG on depressive-like behaviors

Repeated panic-like episodes alone led to the increase of the immobility time in the FST, but had no effects on saccharin preference. Kindling alone did not affect performance in either FST or SPT. The combination of BLA kindling and panic-like reactions induced by DPAG stimulations further exacerbated despair-like behavior in the FST (Fig. 4A), and induced anhedonia-like state which was evident as the disappearance of saccharin preference (Fig. 4B).

4. Discussion

The main findings of this study are that intermittent kindling of limbic seizures and repeated panic-like episodes (a) have bidirectional mutually exacerbating effects, and (b) precipitate depressive-like behavioral impairments.

4.1. Bidirectional connection between kindling and PLB

The combination of repeated BLA and DPAG stimulations produced more profound increase of the BLA excitability, as compared with kindling alone. Furthermore, as DPAG stimulation by itself did not modify afterdischarge properties, the observed hyperexcitability was not due to non-specific additive effect of the electrical stimulations of BLA and DPAG. Rather, repeated panic-like episodes induced by repeated DPAG stimulations, were likely to contribute to the exacerbation of the BLA kindling. It has been shown that repeated stimulation of the DPAG over 30 days in adult rats increase the excitability of the amygdala, judging by the AD properties, and facilitate kindling of the amygdala. Conversely, neither 10, nor 20 days of DPAG stimulation had any such effect [31]. In this regard, our findings are congruent with earlier studies.

At the same time, combined stimulations of BLA and DPAG increased the susceptibility to most of the panic-like behavioral responses. Again, there was likely specific synergy with this regard, since neither BLA, nor DPAG stimulation by itself lowered the threshold for various types of PLB. It is worth noting the absence of “kindling” of panic-like reactions upon the repeated DPAG stimulations. Therefore, the deterioration of panic behavior during the BLA+DPAG stimulations apparently reflected specific facilitated recruitment of relevant neuronal circuits.

In summary, our results indicate that there is specific bidirectional interaction between mechanisms that underlie seizures emanating from limbic structures on the one hand, and mechanisms that determine the emergence of panic anxiety on the other hand. These results reflect clinical observations regarding the high degree of comorbidity between temporal lobe epilepsy and PD [32].

4.2. Kindling epileptogenesis and PLB precipitate depressive-like impairments

Repeated panic-like reactions by themselves were sufficient to induce despair-like behavior, which was further exacerbated when panic-like episodes were intermitted with kindling. In addition, the kindling-PLB combination precipitated anhedonia, thus reproducing two core features of depression.

While psychological factor undoubtedly play a role in depression associated with epilepsy and anxiety [33], the fact that the exacerbation, and even induction of depressive-like behaviors could be reproduced in the animal system, points towards the existence of a neurobiological substrate of the epilepsy-anxiety-depression interaction. With regard to the presence of despair-like behavior after DPAG stimulations, the comorbidity between PD and depression, independent of epilepsy, has been well established [18, 19, 34, 35]. However, experimental studies of PLB and depression comorbidity are scarce. Several reports suggest antagonistic, rather than agonistic relationship between PLB induced by DPAG activation

and depressive-like behaviors [13]. The possible explanation for the differences between the cited and our study is the interference of additional phenomena on the PD-depression comorbidity such as the maternal separation during neonatal age of the animals. Nevertheless, our results are more congruent with clinical findings [18, 19, 34, 35].

Several studies have shown the presence of depressive-like behavior after kindling of the hippocampus (84 trials administered during one day) [36], and of BLA (40 trials administered over two days) [37]. In our study, kindling alone did not produce depressive-like behaviors. The most obvious difference between ours and the cited studies is the number of kindling trials. The question as to whether such a high number of stimulations as used in the cited reports could also induce PLB, and whether the latter contribute to the emergence of depressive-like impairments, remains to be answered.

In conclusion, our experiments offer experimental evidence for the comorbidity between PD and epilepsy, and further, for the triple-morbidity further between epilepsy, PD and depression. This system can be used for studying neuroanatomical and physiological substrates of these comorbidities, through the interrogation of relevant neuronal circuitries.

Acknowledgments

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Abbreviations

ADD	Afterdischarge duration
ADT	Afterdischarge threshold
BLA	Basolateral amygdala
DPAG	Dorsal periaqueductal gray
DR	Dorsal raphe nucleus
DRD	Dorsal region of DR
DRVL	DR ventrolateral
EEG	Electroencephalography
FST	Forced swimming test
GAD	generalized anxiety disorder
PA	Panic attacks
PD	Panic disorder
PLB	Panic-like behavior(s)
QoL	Quality of life
SEM	Standard error of mean

SPT	Saccharin preference test
VLPAG	Ventrolateral periaqueductal gray

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Highlights

- Rats were stimulated in BLA & DPAG to induce epileptic state & PD susceptibility.
- Pre-& post-stimulation, ictogenesis was examined, & depression was characterized.
- BLA+DPAG stimulations increased both seizure severity and PD susceptibility.
- Bidirectional detrimental effects of kindling and PD contribute to depression.

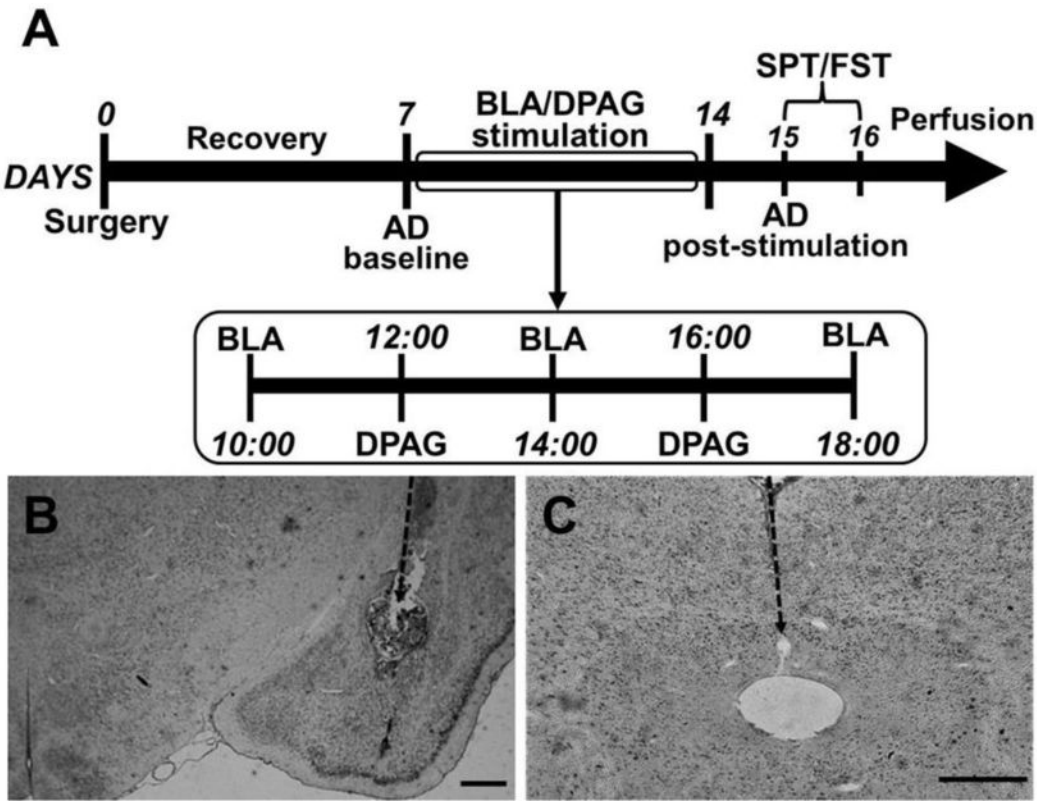

Figure 1.

Experimental protocol. **A**. Day 0, stereotaxic surgery for the implantation of the electrodes, followed by seven days of recovery. The afterdischarge tests were performed before (day 7) and 24 h after (day 15) the electrical stimulations. BLA and DPAG stimulations were induced over seven consecutive days (the box shows the daily stimulation protocol). After completion of electrical stimulations (during days 15 and 16), the behavioral tests (SPT and FST) were carried out. Photomicrographs indicate the trajectory (dashed line) and the implantation site (arrowhead) of the electrodes, which were located in the BLA (**B**) and DPAG (**C**) as was expected. This was achieved in 100% of the experimental subjects. Scale bar = 500 μm.

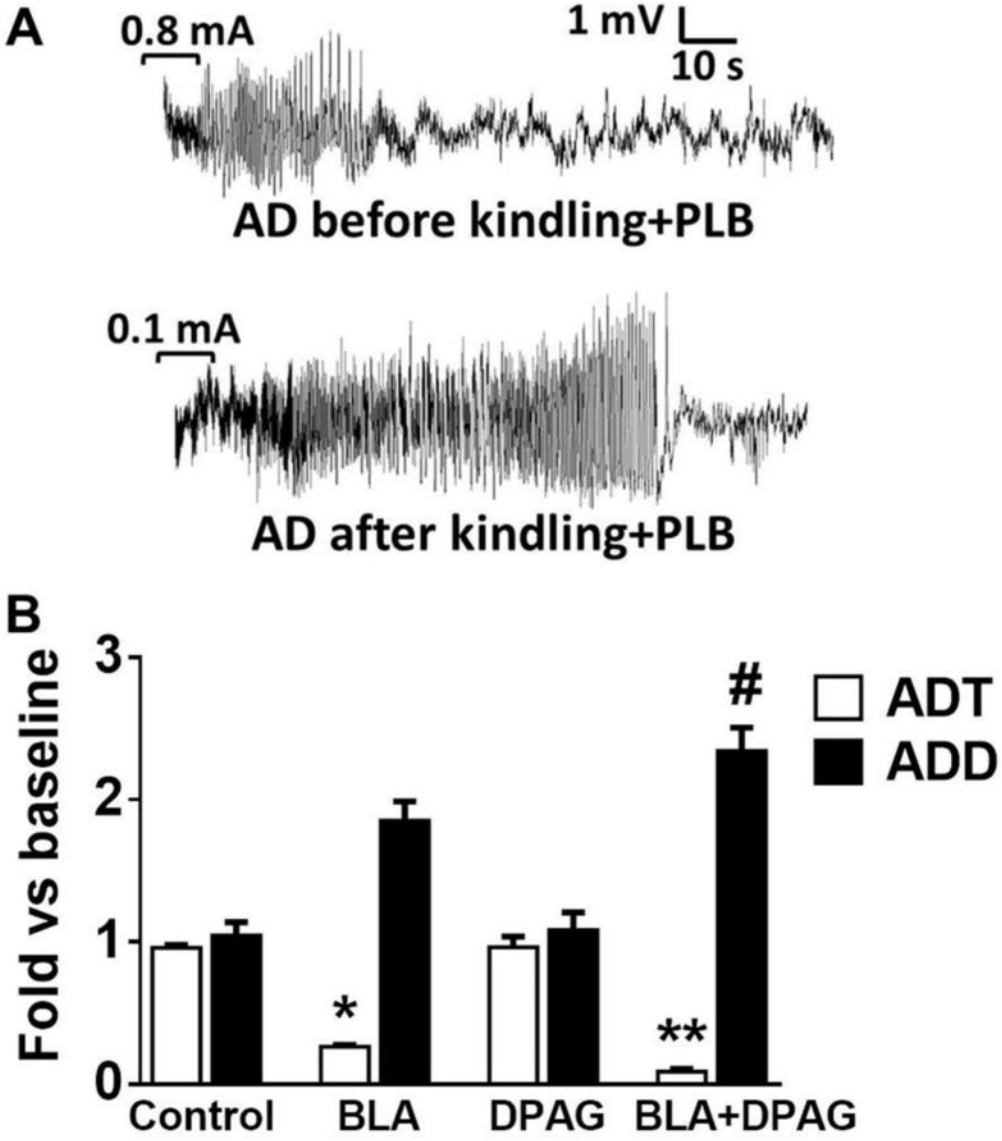

Figure 2.

BLA excitability after intermittent BLA and DPAG stimulations. **A**. Sample EEG recordings obtained before (above) and 24 hours after (below) stimulations from a BLA+DPAG stimulated rat in response to the threshold stimulation. The afterdischarge electrographic signal observed after the application of the electrical stimulation protocol corresponds to stage 4–5 behavioral seizures. Horizontal brackets and the numbers on the first part of each recording indicate the stimulus train and the applied current. **B**. Both BLA-only and BLA +DPAG stimulated groups showed a reduced ADT as compared with the baseline. These treatments increased the ADD as compared with the baseline. In both cases, the BLA +DPAG stimulation produced a more robust effect. Data are presented as mean ± SEM and are expressed as fold-changes vs. baseline values. *p<0.05, **p<0.001 vs. ADT-control group; #p<0.01 vs. ADD-control group (Kruskal-Wallis one-way ANOVA followed by Dunn's test).

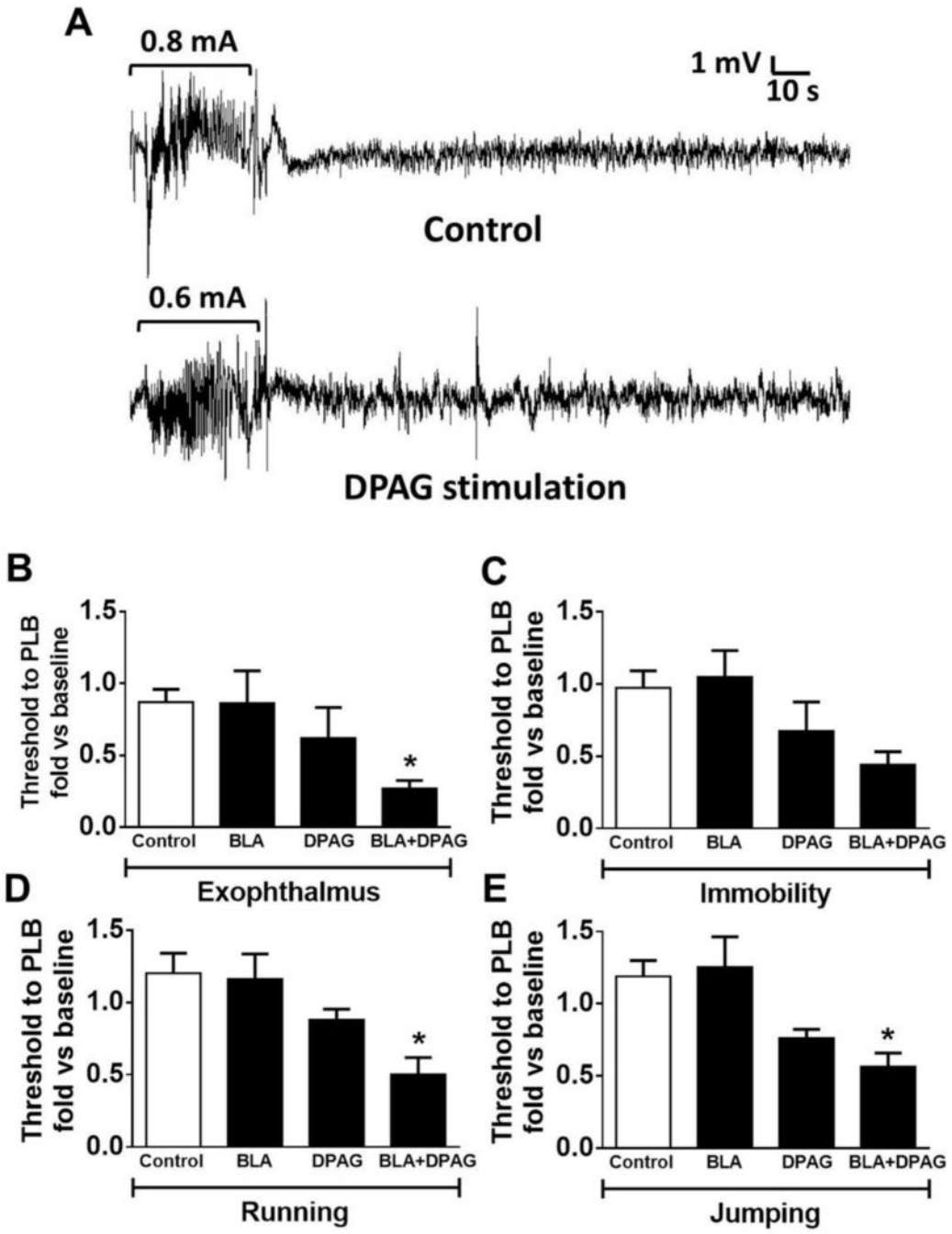

Figure 3.

Panic-like behaviors after intermittent BLA and DPAG stimulations. **A**. Sample post-stimulation EEG recordings showing that neither control (above), nor DPAG stimulation (below) produced electrographic seizures in response to the threshold stimulation to induce the most severe PLB (jumping). Horizontal brackets and the numbers indicate the stimulus train and the applied current. The animals that underwent BLA and DPAG concurrent electrical stimulations exhibited a significant decrease of exophthalmus (**B**), running (**D**) and jumping (**E**) threshold compared to the baseline. However, the immobility (**C**) behavior did

not change after none of the treatments. *p<0.01 vs. control group. Data are presented as mean ± SEM and expressed as fold-changes vs. baseline (Kruskal-Wallis one-way ANOVA followed by Dunn's test).

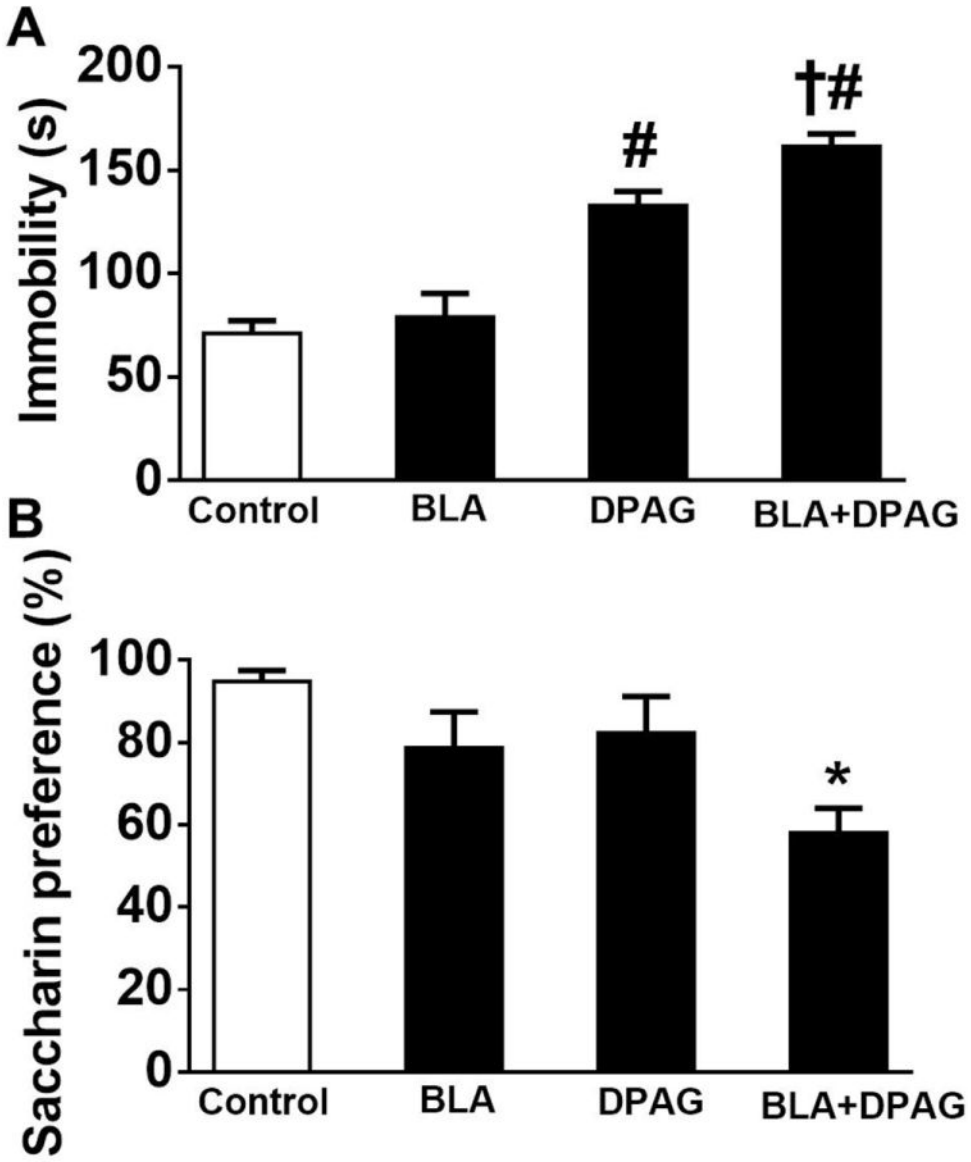

Figure 4.

Depressive-like behaviors after intermittent BLA and DPAG stimulations. **A**. In the animals of both DPAG alone and BLA+DPAG groups an increase of the immobility time during FST was observed. Notably, the combined induction of BLA and DPAG stimulations caused a more intense despair/hopelessness state. Data are presented as mean ± SEM. $^{\dagger}p<0.05$ vs. DPAG group; #$p<0.001$ vs. the rest of the groups (One-way ANOVA followed by Holm-Sidak's test). **B**. Only the rats of the BLA+DPAG group showed a decreased saccharin preference as a sign of anhedonia. Data are presented as mean ± SEM. *$p<0.01$ vs. control group (Kruskal-Wallis one-way ANOVA followed by Dunn's test).