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Research report

High-frequency stimulation of the dorsolateral periaqueductal gray and ventromedial hypothalamus fails to inhibit panic-like behaviour

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ABSTRACT

Electrical stimulation of the dorsolateral periaqueductal gray (dlPAG) and one of its target structures, the ventromedial hypothalamus (VMH), produces a typical behaviour in rats consisting of vigorous running and jumping which is known as "escape behaviour". Escape behaviour in rodents closely mimics panic attacks in humans. Since electrical stimulation at higher frequencies generally inhibits the stimulated region, we tested in this study the hypothesis that deep brain stimulation (DBS) of the dlPAG and VMH at higher frequencies (>100 Hz) would not induce escape behaviour. More specifically, we evaluated whether experimental DBS could be used to inhibit panic-like behaviour. Rats underwent implantation of DBS-electrodes at the level of the dlPAG and VMH and the effects of various stimulation parameters were assessed. In addition, we studied the neural activation pattern resulting from DBS of the dlPAG and VMH using c-Fos immunohistochemistry. We found that stimulation amplitude is the most important stimulation parameter in the induction of escape behaviour. Remarkably, stimulation frequency (1–300 Hz) had no effect on stimulation-induced escape behaviour and therefore it was not possible to prevent the induction of escape behaviour with higher frequencies. The neuronal activation pattern resulting from dlPAG and VMH DBS was similar. These findings suggest that DBS of the dlPAG and VMH induces panic-related behaviours even at higher frequencies.

1. Introduction

The use of stimulation electrodes implanted in the brain to control severely disabling psychiatric and neurological conditions is an exciting and fast emerging area of clinical neuroscience [44,53,60]. Its popularity is directly related to its minimal invasiveness, reversibility (stimulation can be turned on and off), and adjustability (various combinations of stimulation parameters are possible). Currently, thousands of patients have been implanted with stimulating electrodes and especially in the field of psychiatric disorders new indications are evaluated. For instance, in case series, deep brain stimulation (DBS) has relieved symptoms in patients with treatment-resistant depression [38] and obsessive-compulsive disorder [1,43].

Before applying DBS for new indications in clinical psychiatry, it is preferred that the effects of DBS are evaluated systematically in animal models. In this respect, DBS could be considered to treat refractory panic and anxiety disorders. Panic disorder (PD) is one of the most frequently encountered anxiety disorder and one out of every seventy five people worldwide experiences panic attack (PA) once in their lives [42]. Panic attacks are characterized by an acute moment of intense fear or psychologically distress with a sudden onset rapidly building to a peak level and are usually accompanied by somatic and psychological symptoms associated with a sense of impending danger and an urge to escape from the susceptible risk condition [15,26,32]. Panic disorder is recognized as a disabling condition and its treatment includes pharmacologic and behavioural therapies as well as combinations of these strategies [3,5,24]. In some cases, patients do not respond to these treatments

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and experience a chronic course of disease burden, usually together with their families [23,46,48].

In preclinical experiments, electrical stimulation of the dorsolateral area of the Periaqueductal gray (PAG) and one of its target structures, the ventromedial hypothalamus (VMH), has been used to study the mechanism of PA. Electrical stimulation of these areas produces a typical behaviour consisting of vigorous running and jumping in rats [13,29,31,50,54]. This behaviour, also known as escape behaviour, is accompanied by strong emotional and autonomic activation [4,33,52]. Escape behaviour in rodents closely mimics PA in humans [18,50]. The neural circuit responsible for stimulation-induced escape behaviour has been partly identified. Several brain structures interconnected with the dorsolateral periaqueductal gray (dlPAG) have been reported to show changed neuronal activity due to electrical or chemical stimulation. For instance, electrical and chemical stimulation induced significant c-Fos expression in the amygdala, thalamus, and medial prefrontal cortex (prelimbic, infralimbic and cingulate areas) [11,21,59]. Another region that is strongly activated by dlPAG stimulation is the ventromedial hypothalamus (VMH) [11,17].

In previous experiments, several stimulation paradigms have been applied to manipulate the dlPAG and VMH, generally aiming to evoke escape behaviour [8,30,31,49]. Therefore, only stimulation parameters inducing escape behaviour were investigated. Almost all studies focused on the stimulation intensity with a fixed frequency (usually 50–100 Hz) [16,51,58]. The intensities of stimulation varied greatly among studies [12,28,37,54]. In this era in which electrical brain stimulation has become a widely applied technology in preclinical studies, a systematic analysis of effects of stimulation parameters on escape behaviour is needed using a stimulation set up similar to clinical DBS [6,7,34,55]. The effect of stimulation frequency is of particular importance, since this determines whether a brain region is activated or inhibited [20,40,55]. For instance, it has been demonstrated repeatedly that high-frequency stimulation (HFS) inhibits the target by reducing its neuronal firing by a mechanism involving either a deporalization blockage, neurotransmitter depletion, and/or stimulation of presynaptic terminals with neurotransmitter release [7,22,36,41]. In this study, we examined the behavioural and neuronal effects of dlPAG- and VMH DBS stimulation, and tested the hypothesis that HFS could be used to inhibit panic-like behaviour. Furthermore, the effect of stimulation parameters on neural activation patterns was examined in the forebrain using c-Fos immunohistochemistry.

2. Materials and methods

2.1. Subjects

Subjects were Wistar male rats ($N = 24$, 12 weeks old, bred and housed at the Central Animal Facility of Maastricht University, Maastricht, the Netherlands). Rats had an average body weight of 300–350 g at the time of surgery. They were housed individually in standard cages on sawdust bedding in an air-conditioned room (about 20 °C) under a 12/12-h reversed light/dark cycle. Food, standard laboratory chow (Hopefarms, Woerden, The Netherlands), and water were available ad libitum. All experimental procedures were approved by the Animal Experiments and Ethics Committee of Maastricht University.

2.2. Experimental groups

Rats were randomly assigned to one of three following experimental groups: A. Control ($n = 6$); B. dlPAG stimulation ($n = 10$); C. VMH stimulation ($n = 8$).

2.3. Surgical procedure

A detailed description of the surgical procedure and the electrodes has been provided earlier [55,57]. In brief, the rats were anesthetized throughout the entire procedure using a combination of ketamine (90 mg/kg s.c.) and xylazine (10 mg/kg s.c.). Rats were placed in a stereotactic apparatus (Stoelting, Wood Dale, USA; model 51653). After making a burr hole in the skull, rats of groups B and C received implantation of the electrodes at the level of the dlPAG (coordinates from Bregma: AP, −7.6; ML, 0.7; and V, −4.8; approached with a coronal angle of 10°) and VMH (coordinates from Bregma: AP, −2.5; ML, 0.5; and V, −9.5), respectively. A construction of one gold-plated needle-like electrode with an inner wire of a platinum–iridium combination (Technomed, Beek, The Netherlands), with a tip diameter of 50 μm and a shaft diameter of 250 μm, was employed in this experiment. The electrodes were fixed in position using dental cement (Heraeus Kulzer, Hanau, Germany). Rats had a one-week recovery period.

2.4. Behavioural evaluation

Rats were evaluated in an open field (OF) test. For more details of this test please see Hameleers et al. [27]. In summary, rats were placed in the arena and were connected with an external stimulator through externalized leads. The stimulation started approximately 1 min after the rat was placed in the OF arena. The behaviour of the rats was videotaped (Ethovision®, Noldus Information Technology, Wageningen, the Netherlands). We observed three different types of behaviour during the experimental procedure, consisting of 'no effect', 'freezing' behaviour characterized by piloerection, micturition/defaecation, and immobility, and 'escape behaviour' characterized by rigorous and aimlessly running. The stimulation procedures and behavioural analysis were evaluated by two researchers.

2.5. Deep brain stimulation

For DBS we used a variety of stimulation amplitudes (1–650 μA), and stimulation frequencies (1–300 Hz) at a quasi-random fashion. The pulse width was set at 100 μs since in previous experiments variation in pulse width did not influence behavioural performance [20]. A World Precision Instruments digital stimulator (DS8000, WPI, Berlin, Germany) and a stimulus isolator (DLS100, WPI, Berlin, Germany) were used to deliver the stimuli. Real time verification of the parameters applied during stimulation was obtained using a digital oscilloscope (Agilent 54622D oscilloscope, Agilent Technologies, Amstelveen, the Netherlands). The electrical stimulation was performed with gradual increase of the current amplitude. At each step, stimulation duration was 15 s followed by a stimulation off period of 45 s. The stimulation frequency was set at 50 Hz, and pulse width at 0.1 ms. Behavioural changes during this procedure were observed by two researchers. Freezing behaviour was consistently characterized by immobility (without body movement) accompanied by two distinctive symptoms of increased alertness (head scanning) and piloerection. After cessation of stimulation, freezing disappeared and animals began to move again.

2.6. Histological processing

Two hours after the final electrical stimulation procedure, rats were perfused transcardially with Tyrode (0.1 M) and fixative containing 4% paraformaldehyde, 15% picric acid and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.6). Brains were removed and post-fixed for 2 h followed by overnight immersion in 10%, 20%, and 30% sucrose at 4 °C. Brain tissue was then quickly frozen with CO_2 and stored at −80 °C. Subsequently, the prefrontal regions of the brains were cut serially on a cryostat into 30 μm frontal sections and again stored at −80 °C. c-Fos immunohistochemistry was carried out by using an anti-c-Fos rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (diluted 1: 20 000 in 0.1% Bovine Serum Albumin and Tris Buffered Solution-Triton (TBS-T) solution). After overnight incubation at room temperature on a constant shaker, sections were rinsed with TBS-T, Tris Buffered Solution (TBS), and TBS-T and incubated with the secondary antibody (diluted 1: 400 in biotinylated donkey anti-rabbit biotine; Jackson Immunoresearch Laboratories Inc., Westgrove, USA) for 90 min. Subsequently, the sections were incubated with an avidin-biotin-peroxidase complex (diluted 1:800, Elite ABC-kit, Vectastatin; Burlingame, USA) for 2 h. In between steps, sections were washed with TBS and TBS-T. To visualize the immune complex of Horse Radish Peroxide reaction product, sections were incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB)/nickel chloride ($NiCL_2$) solution (5 ml DAB solution, 5 ml Tris/HCl, 50 μl $NiCL_2$, and 3.35 μl hydrogen peroxide). This reaction was stopped after 10 min by rinsing thoroughly all the sections with TBS. All sections were then mounted on gelatin-coated glasses. After dehydrating, all sections were coverslipped with Pertex (HistolabProducts ab, Goteborg, Sweden). Additionally, another series of sections per animal was stained with standard hematoxylin–eosin (Merck, Darmstadt, Germany) to evaluate the localization of the electrode tips.

2.7. Semi-quantitative evaluation of c-Fos immunoreactive cells

Systematic cell counts were performed of c-Fos immunoreactive (c-Fos-ir) cells in the prelimbic (PrL), infralimbic (IL), cingulate (Cg1) and motor (M2) cortices. Photographs of the areas of interest were taken at 4X magnification using an Olympus DP70 camera connected to an Olympus AX70 bright-field microscope (analySIS; Imaging System, Münster, Germany). Our quantification method was similar to a previously reported method, with minor modifications [35]. Four sections from each animal were selected for quantification. The boundaries of the areas of interest were delineated and measured. The same light intensity and threshold conditions were employed for all sections. The counting of the numbers of c-Fos-ir cells was performed using the conventional image analysis program 'Image J' (version 1.38, NIH,

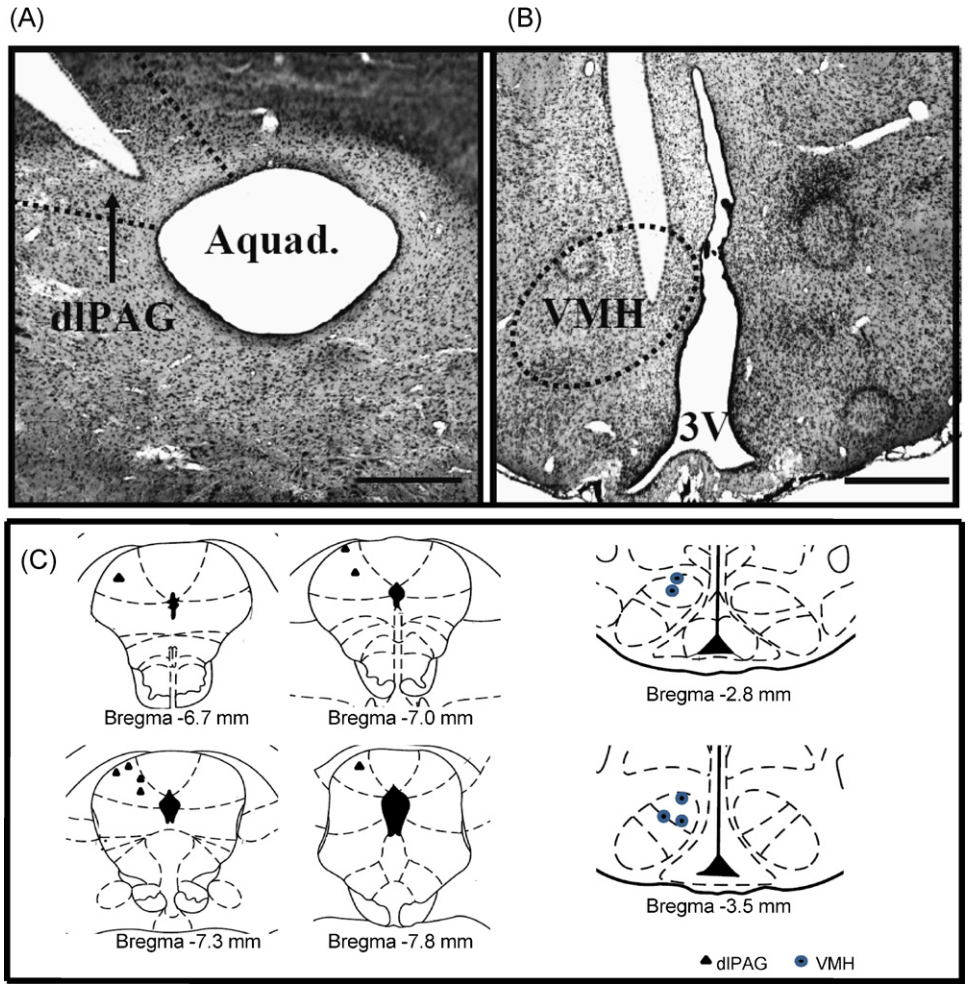

Fig. 1. Representative low-power photomicrographs of a 30 μm-thick frontal section from the brain of a rat subjected to stereotactic implantation of concentric bipolar electrode to stimulate the dlPAG (Fig. A, scale bar = 250 μm) and VMH (Fig. B, scale bar = 500 μm) are shown. Fig. C, Schematic representation of the electrode sites in the dlPAG and VMH according to the Paxinos and Watson atlas, respectively. The symbol (▲) indicates the electrode localization in the dlPAG and (●) in the VMH. Abbreviation: Aquad. = aquaduct of Sylvius, 3V = third ventricle.

USA). A cell was counted as c-Fos-ir if its density was 75% higher than the background density. In addition, artifacts in the sections were excluded from analysis to ensure the accuracy of measurements.

In this study, this method of quantification was preferred to a stereological analysis which is laborious, since the differences were clear and therefore data about relative differences were sufficient for our purposes.

2.8. Statistical analysis

Data are presented as mean ± S.E.M and were analyzed using Analysis of Variance (ANOVA). Data of c-Fos-ir cell counts are expressed as number of c-Fos-ir cells/mm^2. An LSD post-hoc test was used to analyze group differences in more detail. *P*-values lower than 0.05 were considered significant.

3. Results

3.1. Histological evaluation of the electrode localization

Electrode tips were located at the level of the dlPAG in 8 rats of group B, and in 5 rats of group C electrodes were located in the VMH. In two rats of group B and 3 rats of group C, the electrodes were misplaced and these rats were excluded from analysis. The localization of the electrode tip in the PAG and VMH is illustrated in Fig. 1. Based on the evaluation of the hematoxylin and eosin staining, no neuronal damage was observed except for the electrode trajectory.

3.2. Behavioural evaluation

3.2.1. Effect of stimulation parameters

The intensity of the electrical current applied to the dlPAG and VMH of the animals was based on the lowest threshold to induce escape behaviour. The current amplitudes necessary for inducing freezing behaviour and escape behaviour differed significantly between rats with dlPAG- and VMH DBS (F's > 351.13; P's < 0.00). The current intensity applied to the dlPAG and VMH DBS to induce freezing behaviour was 78.57 ± 4.04 μA and 540.00 ± 29.15 μA, respectively, while the current intensity to evoke escape behaviour was 85.71 ± 13.36 μA and 590.00 ± 29.15 μA, respectively (Fig. 2.). After confirming the current amplitudes necessary to evoke escape behaviour, we evaluated the effect of stimulation frequency. We found that each stimulation frequency applied resulted in escape behaviour (Table 1).

3.2.2. Effect of repeated stimulation

Rats that showed escape behaviour in the previous session, showed no escape behaviour when they were stimulated again at their escape threshold on the second day. However, after a longer period of recovery (seven days), rats again showed escape behaviour when stimulating at their escape threshold. Furthermore, the rats which showed escape behaviour due to stimulation,

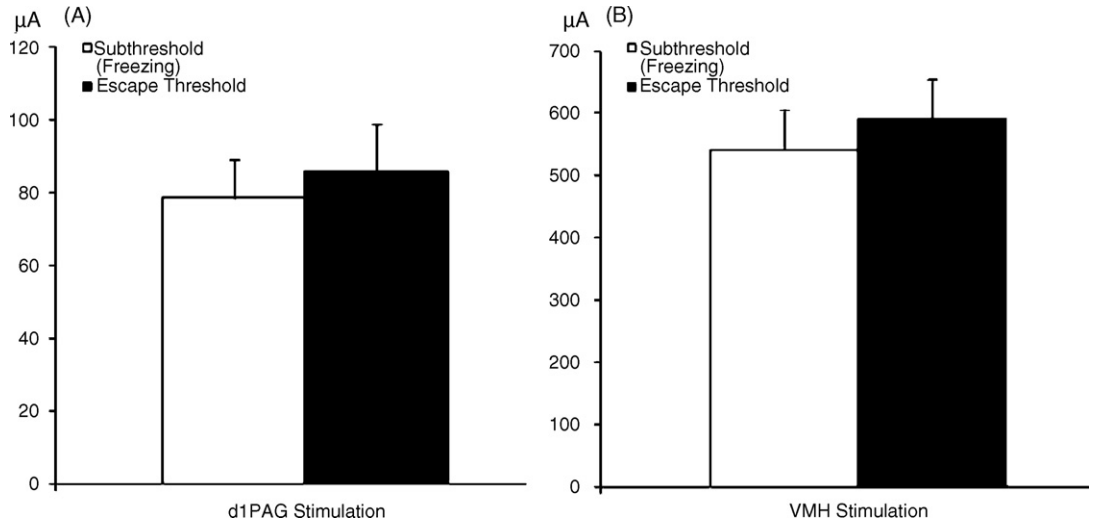

Fig. 2. This figure shows the current amplitudes (μA) necessary to induce freezing-like behaviour and escape reaction. Data represent means ± S.E.M. of dlPAG (Fig A) and VMH stimulation (Fig. B) groups.

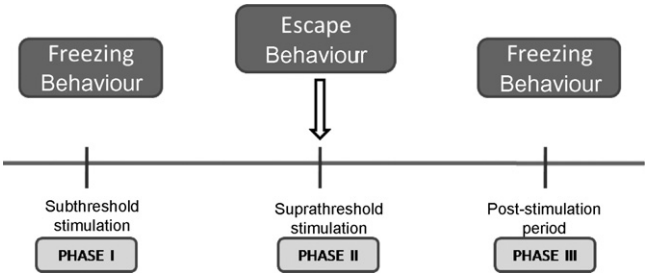

Fig. 3. Behavioural effects of sub-threshold stimulation (phase I), suprathreshold stimulation (phase II) and post-stimulation period (phase III). We found that stimulation below the escape-threshold induced freezing behaviour. In addition, rats showed freezing behaviour when they were placed in OF arena one day after the stimulation experiment.

showed freezing behaviour when they were placed in the OF arena the next day (Fig. 3).

3.3. Evaluation of c-Fos-ir cells

Since numbers of c-Fos-ir cells in the ipsilateral and contralateral regions were not significantly different (P's > 0.05), we pooled these data. DBS of the dlPAG and VMH significantly increased the number of c-Fos-ir cells in the PrL (F's > 53.96, P < 0.00), IL (F's > 90.85, P < 0.00), M2 (F's > 77.93, P < 0.00) and Cg1 (F's > 95.24, P < 0.00) regions as compared to control animals. In all regions, VMH DBS resulted in higher numbers of c-Fos-ir cells as compared to dlPAG DBS (F's > 14.28, P < 0.05) (Figs. 4–6).

4. Discussion

Electrical stimulation of the dlPAG and VMH produced a typical behaviour consisting of vigorous running and jumping in rat, referred as escape behaviour. This behaviour in rodents has been considered a phenomenon closely mimicking PA in humans [18,50]. Our results have revealed that the most important stimulation parameter for this behaviour is the stimulation amplitude. For each subject, a minimum amount of stimulation amplitude (escape-threshold) was necessary to induce escape behaviour. Interestingly, substantially higher amplitudes were required with VMH DBS. In addition, VMH DBS resulted in higher levels of neuronal activation (i.e., more c-Fos-ir cells) in the prefrontal areas associated with panic reactions, which could be related to these high stimulation intensities.

The key finding of this study is that it was not possible to prevent the induction of escape behaviour by PAG and VMH DBS. We have applied a wide range of frequencies which all induced escape behaviour. Even with 300 Hz stimulation with a threshold-amplitude, rats showed escape behaviour. These findings suggest that both dlPAG and VMH are not sensitive for high-frequency stimulation in the context of escape behaviour. This is in contrast with findings in animal models of Parkinson's disease, Huntington's

Table 1
This table shows the effect of stimulation parameters on behaviour

Intensity of current stimulation, amplitude (μA)	Dorsolateral periaqueductal gray							Ventromedial hypothalamus						
	LFS (Hz)				HFS (Hz)			LFS (Hz)				HFS (Hz)		
	1	5	10	50	100	150	300	1	5	10	50	100	150	300
0.25–74 μA	F	F	F	F	F	F	F	NE	NE	NE	NE	NE	NE	NE
75 μA	EB	EB	EB	EB	EB	EB	EB	NE	NE	NE	NE	NE	NE	NE
100 μA	EB	EB	EB	EB	EB	EB	EB	NE	NE	NE	NE	NE	NE	NE
200 μA	EB	EB	EB	EB	EB	EB	EB	NE	NE	NE	NE	NE	NE	NE
400 μA	EB	EB	EB	EB	EB	EB	EB	F	F	F	F	F	F	F
500 μA	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB
600 μA	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB
650 μA	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB	EB

A variety of stimulation parameters consisting of different current amplitudes (1–650 μA) and stimulation frequencies (1–300 Hz) were used. The pulse width was set at 100 μs. Abbreviations: 'LFS': low-frequency stimulation, 'HFS': high-frequency stimulation, 'F': freezing response, 'EB': escape behaviour, and 'NE': no effect.

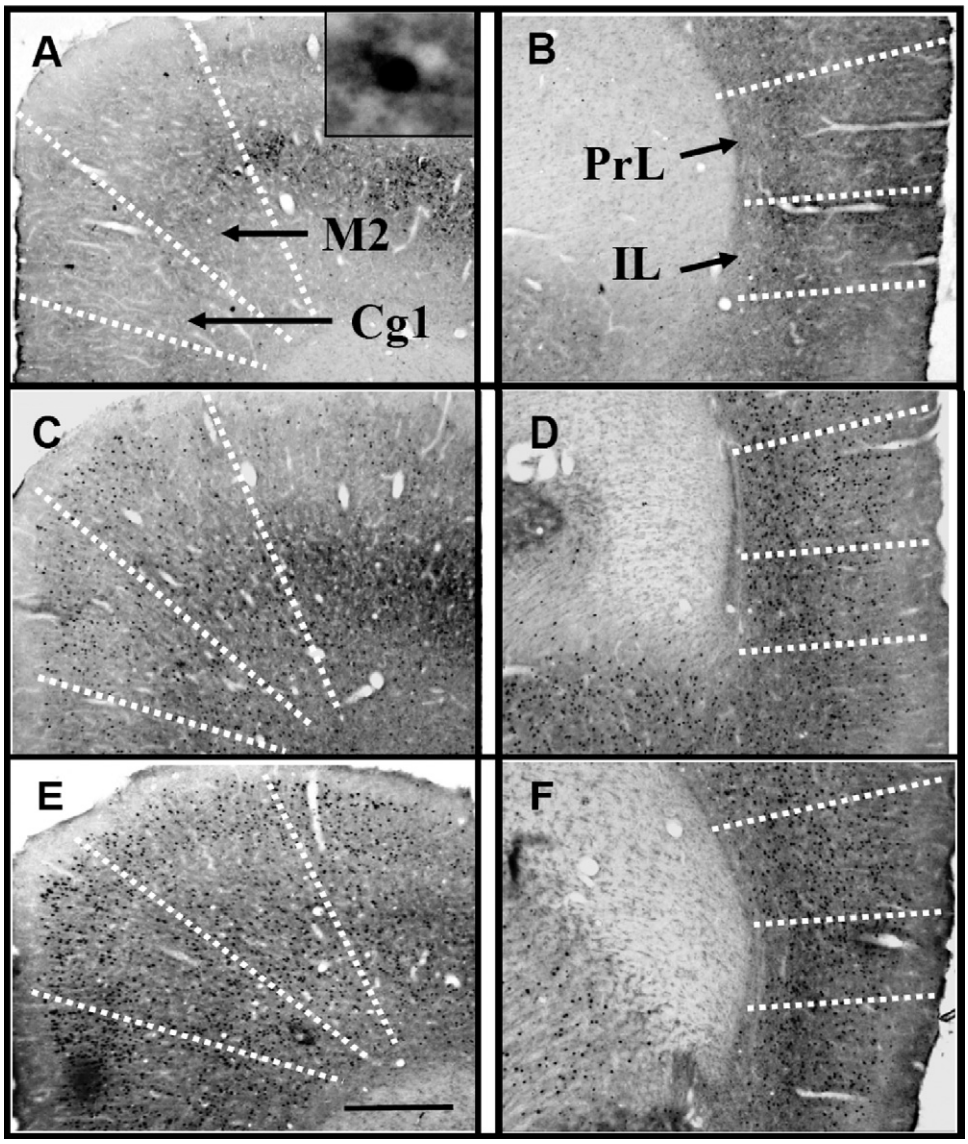

Fig. 4. Representative low-power photomicrographs of 30 μm-thick frontal sections from the brain of a control rat (Figs. A and B), a rat subjected to stereotactic implantation of concentric bipolar electrode to stimulate the dlPAG (Figs. C and D) and VMH (Figs. E and F). Figs A, C, and E at bregma level of 2.20 mm show the level of the Cg1 (Cingulate gyrus 1) and M2 (Motor cortex 2), and Figs. B, D, and F at bregma level of 2.20 mm show the level of PrL (Prelimbic cortex), and IL (Infralimbic cortex). The small dark dots represent c-Fos-ir cells. The inset in A shows a representative high-power photomicrograph of a c-Fos-ir cell. Scale bar = 500 μm.

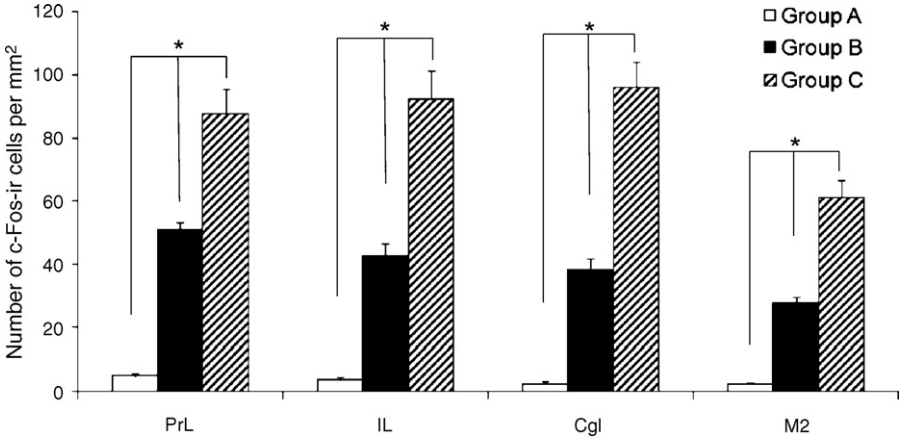

Fig. 5. Grouped data of c-Fos-ir cells/mm^2. Data represents means ± S.E.M. of groups A (control), B (PAG stimulation) and C (VMH stimulation). Abbreviations: PrL: prelimbic cortex; IL: infralimbic cortex; Cg1: cingulate gyrus 1; and M2: motor cortex 2. * indicates significant difference between groups ($P < 0.05$).

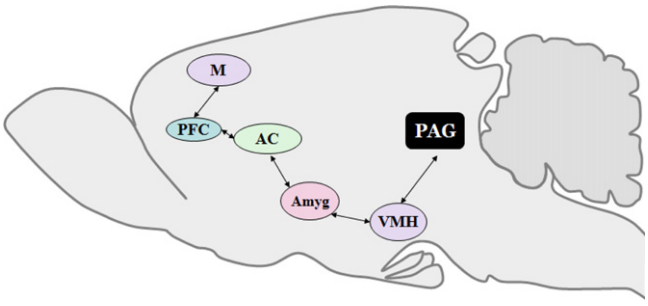

Fig. 6. Schematic illustration of the upstream connections of the periaqueductal gray matter in defensive behaviour based on McNaughton and Corr's two-dimensional defense system [39]. Abbreviations: PAG: periaqueductal gray, VMH: ventromedial hypothalamus, Amyg: amygdala, AC: anterior cingulate gyrus, PFC: prefrontal cortex, and M: motor cortex.

disease or depression [7,41,55,56]. In these models, low frequency stimulation usually deteriorated the condition, whereas HFS was effective in reducing the symptoms. However, an important similarity is the acute effect of dlPAG and VMH DBS on behaviour.

We observed two types of behaviour. Sub-threshold stimulation resulted in freezing behaviour and stimulations at the threshold or above induced escape behaviour. McNaughton and Corr (2004) have introduced the concept of two-dimensional defensive behaviour, categorizing behavioural reactions into avoidable and unavoidable threats such as the escape behaviour and panic-like freezing behaviour [39]. The first dimension describes the two systems of defensive behaviour associated particularly with the PAG and hypothalamus in controlling defensive avoidance (fear) and defensive approach (anxiety). The second dimension illustrates an important hierarchical organization of defensive behaviour. A particular finding in this study, is that rats showed freezing behaviour when they were placed in the OF arena on the next day after a stimulation session. However, when the recovery period was extended, animals returned did not show this behaviour and reacted normally. This finding could be indicative of a fear-conditioning effect with subsequent extinction.

The results of c-Fos immunohistochemistry further establish the involvement of the medial prefrontal cortex in the integration of cognitive-affective information in response to emotional stress [45]. More specifically, the Cg1 plays a role in autonomic functions, such as regulating blood pressure and heart rate [2]. Further, the PrL, and IL cortices appear to be directly involved in cognitive functions, fear expression [14], and in learning and coping with changing contingencies in new tasks or familiar environments [47]. We have also evaluated the c-Fos expression in the motor cortex, especially in the M2 area, to confirm the neuronal substrate of high motor activity associated with escape behaviour. In line with our expectation we found high levels of c-Fos-ir cells in the cortical motor areas. In all regions, VMH DBS resulted in more c-Fos-ir cells as compared to dlPAG DBS. One possible explanation could be the higher amplitudes used with VMH DBS which have influenced neighboring circuits and regions. Another explanation is that the effects of DBS of the VMH are mediated via the dlPAG. This explanation would corroborate the hierarchical neural levels in the two-dimensional defense system (see Fig. 6) [39]. Blanchard & Blanchard also described a similar hierarchy of defensive behaviour [9,10] which was related to the neural hierarchy in the mechanism of defense as proposed by Deakin [19] and Graeff [25]. These theories explained the involvement of different levels of neuroanatomical structures (e.g., septo-hippocampal system, amygdala and hypothalamus) and neurotransmitters (e.g., acetylcholine, serotonin) in facilitating defensive behaviour related to rage and panic, respectively. Conceptually, the PAG has long been known as the primitive defensive structure commanding fight and flight reaction and accordingly higher current amplitudes were necessary with VMH DBS to influence the dlPAG in order to induce such defensive response. Nevertheless, the results obtained with c-Fos immunohistochemistry should be interpreted carefully, since c-Fos is only a general marker of neuronal activity. Consequently, the c-Fos expression may reflect direct and indirect effects, and excitation and inhibition.

In conclusion, the main finding of the present study is that both low- and high-frequency stimulation of the dlPAG- and VMH DBS induced escape behaviour. In addition, different current amplitudes produced different behaviours consisting of freezing and escape. Furthermore, we found simple fear-conditioning with subsequent extinction in the post-stimulation period.

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