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Periaqueductal Grey Stimulation Induced Panic-Like Behaviour Is Accompanied by Deactivation of the Deep Cerebellar Nuclei

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Abstract Until recently, the cerebellum was primarily considered to be a structure involved in motor behaviour. New anatomical and clinical evidence has shown that the cerebellum is also involved in higher cognitive functions and non-motor behavioural changes. Functional imaging in patients with anxiety disorders and in cholecystokinin tetrapeptide-induced panic-attacks shows activation changes in the cerebellum. Deep brain stimulation of the dorsolateral periaqueductal grey (dlPAG) and the ventro-medial hypothalamus (VMH) in rats has been shown to induce escape behaviour, which mimics a panic attack in humans. We used this animal model to study the neuronal activation in the deep cerebellar nuclei (DCbN) using c-Fos immunohistochemistry. c-Fos expression in the DCbN decreased significantly after inducing escape behaviour by stimulation of the dlPAG and the VMH, indicating that the DCbN were deactivated. This study demonstrates that the DCbN are directly or indirectly involved in panic attacks. We suggest that the cerebellum plays a role in the selection of relevant information, and that deactivation of the cerebellar nuclei is required to allow inappropriate behaviour to occur, such as panic attacks.

Keywords Cerebellum · Deep brain stimulation ·
Periaqueductal grey · Ventromedial hypothalamus ·
Escape behaviour

Abbreviations

CCAS	Cerebellar cognitive affective syndrome
OCD	Obsessive-compulsive disorder
CCK-4	Cholecystokinin tetrapeptide
PAG	Periaqueductal grey
DBS	Deep brain stimulation
dlPAG	Dorsolateral periaqueductal grey

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VMH	Ventromedial hypothalamus
DCbN	Deep cerebellar nuclei
OF	Open field
FN	Fastigial nucleus of the cerebellum
DN	Dentate nucleus of the cerebellum
IN	Interposite nucleus of the cerebellum
c-Fos-ir	c-Fos immunoreactive
PC	Purkinje cell

Introduction

The cerebellum has long been considered to be predominantly involved in motor coordination and control [1, 2]. The last two decades, however, it has become increasingly clear that the cerebellum is also involved in behaviour and cognition [3, 4]. Schmahmann and Sherman described the cerebellar cognitive affective syndrome consisting of impairment of executive functions, difficulties with spatial cognition, changes of personality including disinhibited or inappropriate behaviour and language deficits [5]. Recent neuroanatomical and functional imaging research has pointed towards a role for the cerebellum in neuropsychiatric diseases such as autism, schizophrenia and mood and anxiety disorders [6–11]. With respect to anxiety disorders, Levinson described the existence of cerebellar-vestibular dysfunction in 94% of patients with various types of anxiety disorders [12]. De Bellis and Kuchibhatla showed that children and adolescents with post-traumatic stress disorder related to maltreatment have significantly smaller cerebelli than paediatric subjects with generalized anxiety disorder and healthy subjects [13]. Several groups have shown structural and functional differences in the cerebellum of patients with anxiety disorders compared to control subjects [14–22]. In patients with obsessive-compulsive disorder (OCD), a disorder characterised by anxiety, an increase in grey matter was found in the anterior cerebellum [22]. Using functional imaging, Busatto et al. found an increased activation in the superior cerebellum of OCD patients compared to control subjects [14]. Another group showed that after treatment, OCD patients showed a decreased activation in the cerebellum compared to pretreatment scans [15]. In contrast, Nabeyama et al. found decreased activation in untreated OCD patients compared to control subjects, which increased after successful treatment of these patients [16]. In patients with panic disorder, an increased activation of the cerebellum was found when compared to control patients [20]; this activation decreased again after successful treatment of these patients [21]. In healthy individuals in whom panic attacks were induced by cholecystokinin tetrapeptide (CCK-4), an increased activation of the cerebellum was found, especially in the vermis [23–26]. However, the significance of these findings is not yet clear.

Panic disorder is one of the most frequently encountered anxiety disorders [27]. Panic attacks consist of an acute, sometimes unexpected onset of intense fear, accompanied by a variety of physical symptoms [27]. Treatment usually includes behavioural and pharmacological therapies, either alone or in combination. It is thought that panic attacks may be the result of an abnormally sensitive fear network [28]. The periaqueductal grey (PAG) is known to be part of this fear network which further consists of the central nucleus of the amygdala, ventromedial hypothalamus (VMH), hippocampus and other brainstem regions [28]. Deep brain stimulation (DBS) in rats of the dorsolateral periaqueductal grey (dlPAG) and one of its target structures, the VMH has been shown to elicit a typical behaviour consisting of vigorous running and jumping [29]. This behaviour, also known as escape behaviour, is accompanied by strong emotional and autonomic activation, and thereby mimics panic attacks in humans [29, 30].

In the present study, we used DBS of the dlPAG and the VMH as a model of panic attack to evaluate the involvement of the deep cerebellar nuclei (DCbN). In a previous study, we have found that increased impulsivity in rats was accompanied by a deactivation of the DCbN [4]. These findings suggested that the cerebellum plays a role in regulating appropriate behaviour. Based on their findings in a patient with pathological laughter and crying, Parvizi et al. hypothesized that the cerebellum adjusts behavioural responses to the correct situational context of a stimulus, and that when the cerebellum operates on the basis of incorrect information, this could lead to inappropriate or even chaotic behaviour [31]. We hypothesized that deactivation of the DCbN was needed for pathological behaviour to occur. In line with this, here we predicted to find again a deactivation of the DCbN in rats with panic-like behaviour. Panic attacks consist of an inadequate response to a stimulus. Possibly the cerebellum also plays a role in regulation of the response to these stimuli by selection of relevant information. Deactivation of the DCbN may then be linked to an inadequate response to a certain stimulus by disabling this selection process. To analyze activation patterns, we have used c-Fos immunohistochemistry. We focused on the DCbN as these are the output structures of the cerebellum. c-Fos was chosen as it is an immediate early gene which is considered to reflect neuronal activation [32, 33]. The level of c-Fos protein is maximal about 2 h after administration of a stimulus and disappears again after 4 to 8 h even if the stimulus is continued [32]. Therefore, the c-Fos expression found 2 h after a specific stimulus in controlled conditions can be considered to result directly from the stimulus, and this expression can be used to identify brain areas influenced by this stimulus [33].

Materials and Methods

Animals

The animals used were male albino Wistar rats ($n=15$, 12 weeks old, bred and housed at the Central Animal Facility of the Maastricht University, the Netherlands), with an average body weight of 300–350 g at the time of surgery. Rats were housed individually in standard cages with sawdust bedding in an air-conditioned room under a 12/12-h reversed light/dark cycle. Food, standard laboratory chow (Hopefarms, Woerden, the Netherlands) and water were available ad libitum. All experiments were approved by the Animal Experiments and Ethics Committee of Maastricht University.

Surgical Procedure

Rats were randomly assigned to one of the following three experimental groups: control (no surgery), dlPAG DBS or VMH DBS. A detailed description of the surgical procedure was reported previously [29, 34]. In brief, animals were anaesthetized throughout the entire procedure using a combination of ketamine (90 mg/kg) and xylazine (10 mg/kg) injected subcutaneously. Rats were placed in a stereotactic apparatus (Stoelting, Wood Dale, IL, USA; model 51653). After making a burr hole, rats received implantation of a unilateral electrode at the level of the right dlPAG (coordinates from Bregma, AP=−7.6 mm, ML= 0.7 mm and V=−4.8 mm; approached with a coronal angle of 10°) and the right VMH (coordinates from Bregma, AP=−2.5 mm, ML=0.5 mm and V=−9.5 mm). A construction of one electrode with an inner wire of a platinum–irridium combination (Technomed, Beek, the Netherlands), with a tip diameter of 50 μm and a shaft diameter of 250 μm, was implanted in this experiment [35]. The electrodes were fixed in position using dental cement.

Deep Brain Stimulation

After a recovery period of about 1 week, all animals underwent a first stimulation session to determine the escape threshold. The stimulation amplitudes were gradually increased until escape behaviour was observed. At each step, stimulation duration was 15 s followed by a period without stimulation of 45 s. The stimulation frequency was set at 50 Hz and pulse width at 0.1 ms based on previous experiments [29]. 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). After confirming the current amplitudes necessary to evoke escape behaviour, all rats had a period of 2 weeks without stimulation before the final stimulation session in the open field (OF) arena was performed. Control rats were handled and placed in the OF similarly.

Behavioural Evaluation

Rats were evaluated in an OF test. For more details of this test, please see Lim et al. [29]. 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, using the current amplitudes previously eliciting escape behaviour. The behaviour of the rats was videotaped (Ethovision, Noldus Information Technology, Wageningen, the Netherlands).

Histological Processing

Two hours after the final stimulation session, rats were deeply anesthetized and perfused transcardially with Tyrode (0.1 M), followed by a fixative containing 4% paraformaldehyde, 15% picric acid and 0.05% gluteraldehyde in 0.1 M phosphate buffer (pH 7.6). The brains were removed and postfixed for 2 h followed by immersion in 20% sucrose at 4°C until saturated. Brain tissue was then quickly frozen with CO_2 and stored at −80°C. The cerebellum was cut serially into 10-μm sections, which were collected on gelatine-coated glasses. We used a previously published protocol for c-Fos immunohistochemistry [4]. In summary, this staining was carried out by incubating sections two nights with a polyclonal rabbit anti-c-Fos antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).This was followed by overnight incubation with the secondary antibody (biotinylated donkey anti-rabbit, Jackson Immunoresearch Laboratories Inc., Westgrove, PA, USA). Subsequently, the sections were incubated with an avidin–biotin–peroxidase complex (Elite ABC-kit, Vectastatin; Burlingame, CA, USA) for 2 h. To visualize the immune complex of horseradish peroxide reaction product, sections were incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB)/nickel chloride ($NiCl_2$) solution for 10 min. After dehydrating, all sections were cover-slipped with Pertex (HistolabProducts ab, Goteborg, Sweden). Additionally, for each animal, the area of the dlPAG and VMH was cut into 30-μm sections and stained with standard haematoxylin–eosin (Merck, Darmstadt, Germany) staining to locate the electrode tips.

Stereological Quantification of c-Fos Immunoreactive Cells

Stereological analysis was performed with a stereology workstation (CAST-GRID-Computer Assisted Stereological

Toolbox-Olympus, Denmark). After exactly tracing boundaries of the fastigial nucleus (FN) and dentate nucleus of the cerebellum (DN) on microscopic video images displayed on a monitor, numbers of c-Fos immunoreactive (c-Fos-ir) cells in both nuclei were evaluated with the modified Abercrombie cell counting method [36]. All neurons of which the cell bodies gave a positive reaction within an unbiased counting frame distributed in a systematic-random fashion throughout the delineated regions were counted. Estimated numbers of neurons were calculated from the numbers of counted neurons and the corresponding sampling probability. The numerical density (N_V) of c-Fos-ir cells in the nuclei was estimated using the formula $N_V = (N_A/T) \cdot (T/(T+D))$ [36]. In this formula, N_A, T and D are the positive cell number in the unbiased counting frame, the section thickness and the mean particle diameter, respectively. To make an acceptable biological comment on any subject, one should estimate total number of cells rather than numerical density of cells in the interested region. For this reason, we estimated the volume (V) of each cerebellar nucleus by multiplying the total area of each nucleus that is seen in the whole section of cerebellum (ΣA) with the mean section thickness (T), as shown in this formula $V = \sum A \cdot T$ [37]. The total number of cells in a nucleus was estimated by the formula $N = N_V \cdot V$.

Semiquantative Analysis of c-Fos-Immunoreactive Cells

Analysis of the c-Fos-ir cells in the interposite nucleus of the cerebellum (IN) was performed using a previously described semiquantitative method [4]. In summary, photographs of the IN were taken at ×4 magnification using an Olympus DP70 camera connected to an Olympus AX70 bright-field microscope (analySIS; Imaging System,

Münster, Germany). The same light intensity and threshold settings of the camera were employed for all sections. For each animal, we selected two sections of the IN for quantification. The boundaries of the IN were delineated and the area measured. The counting of the c-Fos-ir cells was performed using the image analysis program 'Image J' (version 1.38, NIH, USA). A cell was counted as c-Fos-ir if its density was 65% higher than the mean background density of that section. Artefacts in the sections were excluded from analysis to ensure the accuracy of measurements. The number of c-Fos-ir cells per square millimetre was calculated.

Statistical Analysis

The data of the c-Fos counts were analysed using the Kruskal–Wallis test for non-parametric data. Non-parametric testing was chosen due to small group sizes. A value of $p < 0.05$ was considered significant. Post hoc analysis was performed using the Mann–Whitney test. Since we expected a deactivation in the stimulated groups, we used a one-tailed procedure.

Results

Histological Evaluation of the Electrode Localisation

In all animals included in this study, the electrode tips were positioned correctly in the dlPAG and VMH, respectively (Fig. 1). No histological damage was observed except for the electrode trajectory with the standard haematoxylin–eosin staining, suggesting that stimulation with the current stimulation settings did not cause observable tissue damage.
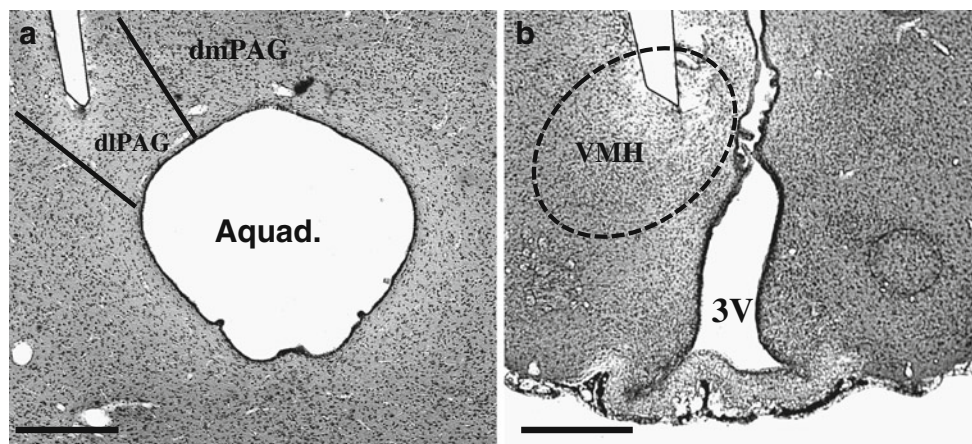

Fig. 1 Representative low-power photomicrographs of 30-μm-thick frontal sections from the brain of a rat subjected to stereotactic implantation of a concentric bipolar electrode to stimulate the dlPAG (**a**, *scale bar*=250 μm) and VMH (**b**, *scale bar*=500 μm). The tips of the electrodes are situated within the respective targets. *Aquad* aqueduct of Sylvius, *dmPAG* dorsomedial periaqueductal grey, *dlPAG* dorsolateral periaqueductal grey, *3V* third ventricle, *VMH* ventromedial hypothalamus

Behavioural Evaluation

The intensity of the electrical current applied to the dlPAG and VMH of the animals was based on the threshold shown to induce escape behaviour. All rats showed 'escape behaviour' characterized by rigorous and aimless running. The current amplitudes necessary for inducing escape behaviour were significantly different between rats with dlPAG- and VMH-DBS (F's>351.13; $p<0.00$). The mean current density applied to the dlPAG to induce escape behaviour was approximately 90 µA and for the VMH, approximately 600 µA.

Evaluation of the Number of c-Fos-Immunoreactive Cells

There was a significant decrease in the amount of c-Fos-ir cells in the dentate nucleus ($H_2=6.343$, $p<0.05$; Figs. 2 and 3) and in the fastigial nucleus ($H_2=6.870$, $p<0.05$; Figs. 2 and 3) after DBS. As expected, we found a lower amount of c-Fos-ir cells in the dentate and fastigial nucleus of the stimulated rats compared to the control rats. Post hoc analysis showed that in the rats stimulated in the dlPAG, the difference with control rats was significant for both nuclei (DN, $U=1.00$, $r=-2.21$; FN, $U=0.00$, $r=-2.45$). When comparing VMH-stimulated rats to control rats, this difference was significant only in the nucleus dentatus ($U=1.00$, $r=-2.02$); the difference in the fastigial nucleus showed a trend towards significance ($U=2.00$, $r=-1.73$).
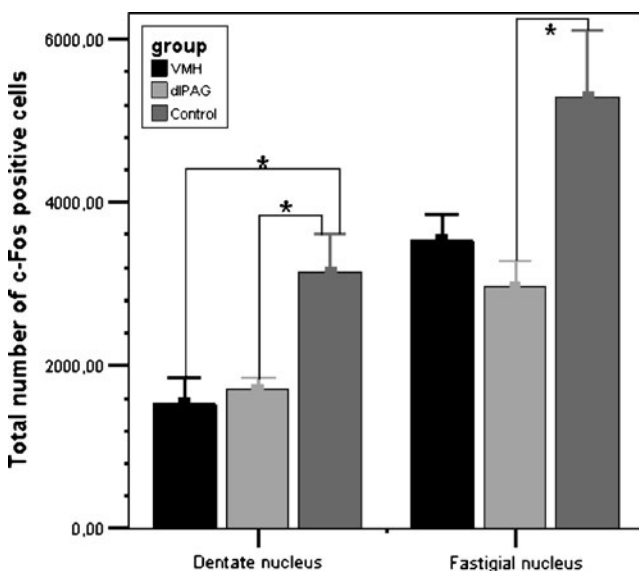

Fig. 2 c-Fos expression in the dentate nucleus and in the fastigial nucleus of the cerebellum. Data are represented as means plus SEM showing control animals, animals stimulated in the dorsolateral periaqueductal grey (dlPAG) and animals stimulated in the ventromedial hypothalamus (VMH). The *asterisk* indicates a significant difference between groups. Note the significantly lower numbers of c-Fos immunoreactive neurons in the groups with animals showing panic attacks evoked by stimulation of the dlPAG and VMH

There was no significant difference in the amount of c-Fos-ir cells between the stimulated groups (DN, $U=7.00$, $r=-0.74$; FN, $U=6.00$, $r=-0.98$).

Analysis of the interposite nucleus (IN) showed a similar trend towards decreased c-Fos expression in the stimulated rats compared to controls (PAG, $U=19.00$, $r=-1.04$; VMH, $U=21.00$, $r=-1.37$). Again, there was no difference between the stimulated groups ($U=36.00$, $r=-0.36$).

Discussion

The objective of our experiment was to use electrical stimulation of the dlPAG and of the VMH in rats as a model of panic attack to study the involvement of the DCbN. We have found that panic-like behaviour was accompanied by a decrease of c-Fos-ir cells in the DCbN, indicating deactivation. c-Fos expression was significantly lower in the DN of both treatment groups and in the FN of the dlPAG DBS group when compared to controls. In the VMH DBS group, c-Fos expression in the FN was lower compared to controls, with a trend towards significance. Using a semiquantitative analysis, the IN showed a similar trend towards decreased c-Fos expression in the stimulated rats compared to controls. This should be considered a preliminary finding since the method of counting was not performed using stereological principles.

There is anatomical and functional evidence supporting the role of the cerebellum in panic. Sakai et al. found a significantly higher glucose uptake in the cerebellum of patients with panic disorder compared to control patients [20]. After clinical improvement of these patients due to cognitive-behavioural therapy, the glucose uptake in cerebellum had decreased [21]. Several groups found increased activation of the cerebellum after CCK-4-induced panic attacks in healthy subjects [24, 25]. This increase was not seen in these subjects during anticipatory anxiety. These studies were not initially designed to analyse changes in the cerebellum, and the changes found are usually unexpected. The findings are often simply mentioned as interesting or surprising, although several groups relate to the role of the cerebellum in fear conditioning as proposed by Sacchetti et al. [38]. Sacchetti et al. reviewed the evidence showing that the cerebellar vermis plays a role in the fear response and in fear conditioning, especially in fear consolidation. The most important changes are thought to take place at the level of the Purkinje cell (PC). It has been shown that several forms of fear conditioning lead to increased PC excitability and an increased firing rate of the PCs. In contrast, heterozygous Lurcher mice, which show early and complete apoptosis of cerebellar PCs, show reduced inhibition to anxiety-provoking aversive areas [39]. In summary, it seems that increased PC activity leads to more fear, and decreased or
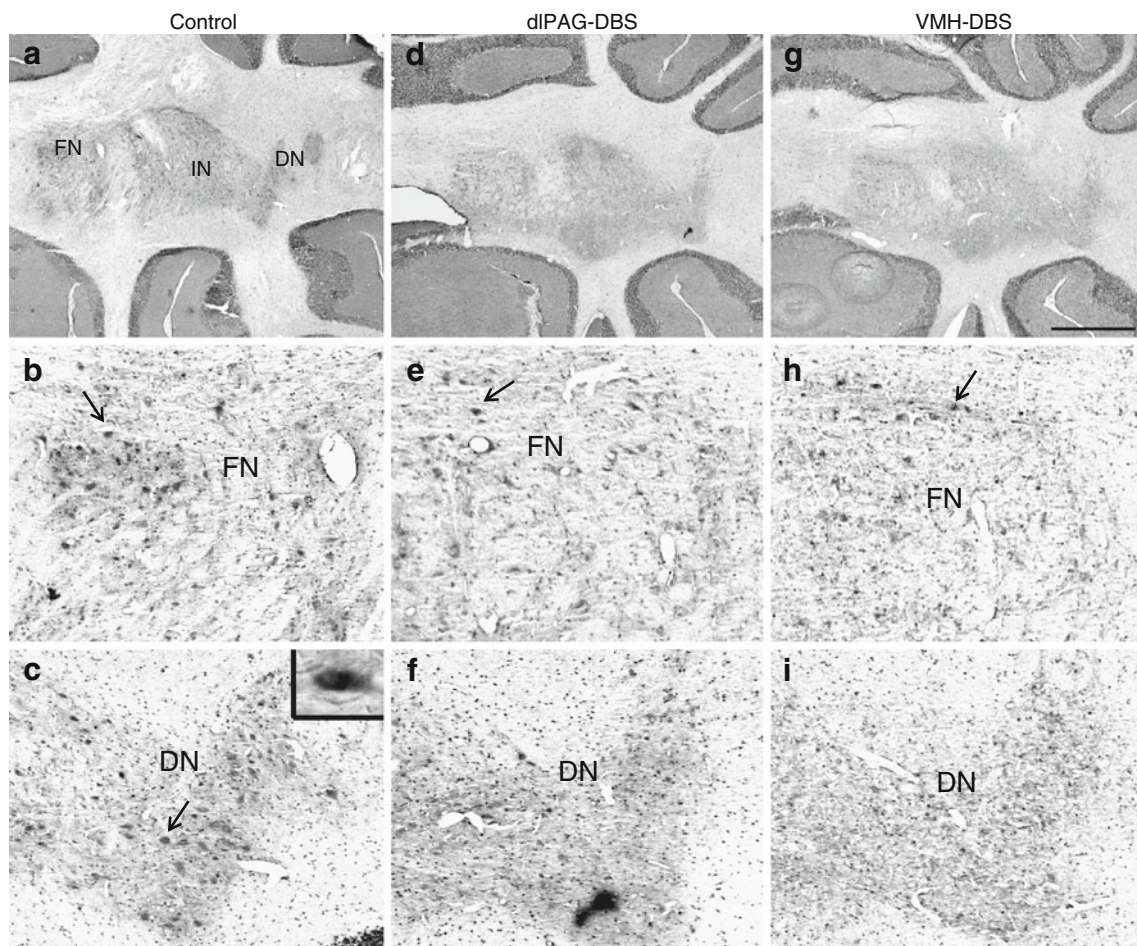
Fig. 3 Representative low-power photomicrographs of c-Fos expression in a 10-µm-thick horizontal section of the deep cerebellar nuclei at Bregma level −6.10 mm (according to the stereotactic rat brain atlas of Paxinos and Watson of 1998) in a sham rat (**a**–**c**), a rat stimulated in the dlPAG (**d**–**f**), and a rat stimulated in the VMH (**g**–**i**). Panels **a**, **d**, and **g** were taken at ×2 magnification, and **b**, **c**, **e**, **f**, **h**, and **i** were taken at high-power magnification. The *arrows* point to a c-Fos-ir cell, represented by a *small dark dot*. The *inset* in **c** shows a representative high-power photomicrograph of a c-Fos-ir cell. *Scale bar* for ×2 magnification power=1 mm. *dlPAG* dorsolateral periaqueductal grey, *VMH* ventromedial hypothalamus, *DBS* deep brain stimulation, *DN* dentate nucleus of the cerebellum, *FN* fastigial nucleus of the cerebellum

absent PC activity to less fear. Functional imaging is designed to analyse activation changes in the cerebral cortex; therefore, it is likely that the activation seen in the cerebellum is also located in the cortex. Increased activation found in functional imaging studies may therefore reflect the increased activity of the PCs. Since PCs are known to have an inhibitory action on the DCbN, these findings are in line with deactivation that we found in the DCbN in the stimulated rats which show panic-like behaviour.

In the cerebellum, the vermis, projecting through the fastigial nucleus, seems to be the most important structure in fear and panic. In the cases described by Schmahmann et al., the vermis was always involved in patients with changes of affect [5]. In early experiments, vermal lesions were shown to attenuate a variety of fear behaviour, whereas vermal stimulation leads to increased fear-related responses [40, 41] Other animal research shows a selective role for the fastigial nucleus in heart-rate conditioning [40, 42, 43]. The vermis also contributes to consolidation of fear memory [44]. There is also evidence of a role of the interposite nucleus and the dentate nucleus in fear. In studies investigating several aspects of fear conditioning, animals with lesions of the dentate and interposite nucleus do not acquire an aversive conditioned response but acquire an appetitive conditioned response [45] and show unaltered heart-rate conditioning [42] and vocalisation indicative of unspecific fear [46]. Furthermore, there are clear bi-directional connections to the hypothalamus from (greatest to least concentration) the dentate nucleus, the interposite nucleus and the fastigial nucleus [47–50], supporting a role of all DCbN in autonomic processes, for example, those

related to fear. In addition, the DCbN have been shown to project to several parts of the fear network: Teune et al. injected tracers in the DCbN in rats and documented projections to the PAG from the fastigial nucleus and the dentate nucleus and, in lesser degree, from the interposite nucleus [51]. Several groups have suggested projections from the fastigial nucleus to the amygdala, hippocampus, septal nuclei and the nucleus accumbens, based on behavioural changes following cerebellar stimulation or lesioning [38, 41, 52]. Berntson and Torello, for example, showed that hyperemotionality caused by septal lesions was largely attenuated by lesions of the fastigial nucleus [53]. An exact anatomical pathway has never been shown [54]. However, these behavioural changes indicate that the involvement of the cerebellum in fear does not merely consist of regulation of an autonomic visceromotor response but that there is a place for the cerebellum in the network regulating fear processing [38]. In a recent review, Stoodley and Schmahmann present a functional somatotopy of the non-motor functions of the cerebellum based on functional imaging [55]. They conclude that the vermis of the posterior lobe seems to be specifically related to emotional processing, whereas activation in the posterior cerebellar hemispheres may be related to the decision-making aspects of the tasks used in the experimental setting [55]. On the other hand, Timman et al. reviewed anatomical evidence for a role of the cerebellum in emotional and cognitive learning, and they conclude that with respect to fear, the vermis, projecting through the fastigial nucleus, contributes to the autonomic and somatic aspects, whereas the posterolateral cerebellar hemispheres, projecting through the dentate and interposite nucleus, play a role in the emotional content of fear processing [40].

In summary, there is ample evidence that all cerebellar nuclei are involved in fear, in which the fastigial nucleus possibly mediates a different aspect of fear than the dentate nucleus and the interposite nucleus. This correlates with our findings in the present study showing a similar deactivation in the dentate nucleus and in the fastigial nucleus, and possibly also in the interposite nucleus. Increased fear is associated with increased PC activity in functional imaging studies, which is hypothesised to lead to inhibition of the DCbN and therefore of cerebellar output. We speculate that the cerebellum plays a role in regulating appropriate behaviour in response to any stimulus, and that a decreased cerebellar output may play a role in emergence of an inappropriate response, such as a panic attack. This decreased output may be in response to incorrect input, as suggested by Parvizi et al. [31]; however, we suggest that the cerebellum also plays a direct role in the selection of relevant information on which an adequate behavioural response is based, and that deactivation of the DCbN then leads to inappropriate behaviour by inhibiting this selection process.

Conclusion

This study supports the hypothesis that the cerebellum is involved in panic attacks. Using DBS of the dlPAG and the VMH in rats as a model of a panic attack, we found that the activity of the DCbN as measured by c-Fos expression decreased significantly in the dentate nucleus and in the fastigial nucleus, and possibly also in the interposite nucleus. In a previous study, we showed that the DCbN are de-activated in rats showing increased impulsivity. In conclusion, deactivation of the cerebellum is associated with inappropriate behaviour such as panic and impulsivity. We suggest that the cerebellum plays a role in the selection of relevant information, thereby inhibiting such inappropriate behaviour.

Conflicts of interest The authors of this manuscript withhold no conflicts of interest.

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