

Reduction in 50-kHz call-numbers and suppression of tickling-associated positive affective behaviour after lesioning of the lateral hypothalamic parvafox nucleus in rats

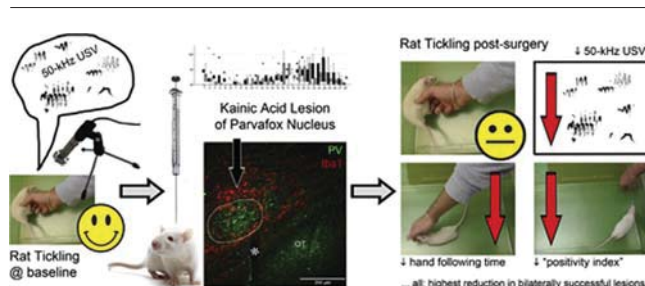
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HIGHLIGHTS

- Wistar rats were tickled before and after excitotoxic lesioning of the lateral hypothalamic parvafox nucleus.
- Ultrasonic vocalisations (USVs) that were emitted during the tickling of rats and their tendency to approach and follow the experimenter's hand were analysed.
- Lesioning was considered successful if the number of parvalbumin-immunoreactive (PV-ir) cells in the area of the parvafox nucleus was reduced beyond a threshold level.
- Rats with bilaterally successful lesions manifested the most profound surgery-associated reduction in the number of 50-kHz USVs and in the tendency to approach and follow the experimenter's hand.
- Positive correlations were found between each of the four investigated parameters.

GRAPHICAL ABSTRACT



The parvafox nucleus is located ventrolaterally in the lateral hypothalamic area (LHA). Its core and shell are composed of neurons expressing the calcium-binding protein parvalbumin (PV) and the transcription factor Foxb1, respectively. Given the known functions of the LHA and that the parvafox nucleus receives afferents from the lateral orbitofrontal cortex and projects to the periaqueductal gray matter, a functional role of this entity in the expression of positive emotions has been postulated.

The purpose of the present study was to ascertain whether the deletion of neurons in the parvafox nucleus influenced the tickling-induced 50-kHz calls, which are thought to reflect positive affective states, in rats. To this end, tickling of the animals (heterospecific play) was combined with intracerebral injections of the excitotoxin kainic acid into the parvafox nucleus.

The most pronounced surgery-associated reduction in 50-kHz call-numbers was observed in the group of rats in which, on the basis of PV-immunoreactive-cell counts in the parvafox nucleus, bilateral lesions had been successfully produced. Two other parameters that were implemented to quantify positive affective behaviour, namely, an approach towards and a following of the hand of the tickling

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experimenter, were likewise most markedly suppressed in the group of rats with bilaterally successful lesions. Furthermore, positive correlations were found between each of the investigated parameters. Our data afford evidence that the parvafox nucleus plays a role in the production of 50-kHz calls in rats, and, more generally, in the expression of positive emotions.

1. Introduction

Evidence that has accumulated over the past decades implicates the lateral hypothalamus in multifarious physiological responses, which include the modulation of not only autonomic and endocrine, but also skeletomotor and even cognitive functions (reviewed in [1]). Anatomically, it can be sub-divided into a preoptic, tuberal and posterior part along the rostrocaudal axis (reviewed in [2]). It contains two major fibre bundles – the fornix and the medial forebrain bundle (MFB) – as well as well-circumscribed cell aggregates (nuclei). The emergence of specific markers for distinct neuronal sub-populations, such as orexin/hypocretin [3], has facilitated a characterization of the functional units within the lateral hypothalamic area (LHA) and their connectivities. We have previously shown that the calcium-binding protein parvalbumin (PV) serves as a marker for yet another neuronal sub-population, which populates the core region of the parvafox nucleus (formerly “PV1-Foxb1 nucleus”). The shell surrounding the core of the nucleus is composed of small neurons, which express the transcription factor Foxb1 [4]. The nucleus is located in the tuberal part of the ventrolateral hypothalamus, between the optic tract and the fornix [5–7]. Experiments in which anterograde tracers, particularly Cre-recombinase-dependent viral ones, have been injected into PV-Cre or Foxb1-Cre mice, have revealed the parvafox nucleus to project mainly to the periaqueductal grey matter (PAG), and, more specifically, to the ventrolateral [8] and the dorsolateral [9] columns. Furthermore, *in-situ* hybridization has disclosed PV-expressing neurons of the parvafox nucleus to be glutamatergic and thus excitatory [10], in contrast to those in most other brain areas, such as the neocortex, the hippocampus and the cerebellum [11].

Hypotheses about possible functions of the parvafox neurons can be generated based on insights gleaned from manipulations in experimental animals, from patients in which alterations in the corresponding region of the LHA are manifested, and from imaging and stimulation studies that have investigated, in animals or humans, the LHA or the PAG, the main target of the axons emanating from the parvafox nucleus.

In humans, hamartomas arising from the lateral tuberal hypothalamus can evoke the onset of gelastic seizures, a form of epilepsy that is characterized by involuntary laughter, which may or may not be accompanied by a pleasant sensation [12,13]. The lateral tuberal nucleus (LTN) of primates shares topographical as well as some neurochemical features in common with the rodent parvafox nucleus [10,14]. Neurons of the LTN manifest pathological alterations in Pick’s disease, in which loss of speech is one of the symptoms [15]. Stimulation of the hypothalamic area can induce vocalization in rats [16], cats [17] and squirrel monkeys [18]. In humans, tickling-induced, involuntary (Duchenne-type) laughter is associated with the activation of several brain regions, including the lateral hypothalamus [19]. The lateral hypothalamus has been ascribed a role in the functioning of the larynx [20] and pharynx [21], as well as in the control of vocalization [22]. Preliminary experiments in which Cre-dependent tracers were injected into the parvafox nucleus have disclosed a projection to the nucleus

retroambiguus [9], which contains premotor interneurons capable to produce the motor actions required for vocalization [23]. In addition to its role in the control of vocalization, the lateral hypothalamus also harbours neuronal representations of reward value [24–26].

The ventrolateral and the dorsolateral PAG are involved in the mediation of two opposing types of emotional coping strategy (passive and active, respectively) [27–29]. The PAG is also implicated in the control of vocalization. As a relay station in the limbic vocalization-control pathway, it serves a gating function in the initiation of involuntarily produced vocalisations ([18,30]; research mainly in monkeys). Although the exact mechanisms remain elusive, the PAG appears to play a role in the production of human laughter [12,31–33]. Functional magnetic resonance imaging has revealed PAG-activity to occur in conjunction with involuntarily produced laughter [19]. Electrical [34–36] or pharmacological [37,38] stimulation of the PAG induces the production of naturally sounding, species-specific vocalisations in all mammalian species that have been thus far investigated. More specifically, the caudal portion of the ventrolateral PAG has been implicated in the induction of relaxed emotional states in rats, cats and monkeys [18,27,39].

In appetitive and neutral situations, rats emit ultrasonic vocalisations (USVs) in a high-frequency range (50-kHz USV, high-frequency calls), whereas in aversive ones, they produce USVs in a low frequency band (22-kHz USV, low-frequency calls) (reviewed in [40]). High-frequency calls are produced by juvenile or adult rats during rough-and-tumble play [41], during mating [41–44], as an expression of reward during voluntary exercise [45], as well as in response to drugs of abuse [46,47], and it is widely accepted today that they reflect a positive affective state [46,48,49]. Consequently, one of their functions appears to be the communication of emotional states to conspecifics [50]. The 50-kHz calls in rats have even been compared to laughter in humans [51,52]. Rough-and-tumble play in rats can be mimicked by tickling [51]. A short period of social isolation has been shown to boost the rate of tickling-induced 50-kHz USVs [51,53] by enhancing the social motivation of the animals. Rats emitting high numbers of tickling-induced 50-kHz USVs have been shown to approach the hand of the tickling experimenter with a short latency period [54]. In addition to conveying information appertaining to affective states, 50-kHz USVs are suggested to fulfil important communicative functions and to be involved in the establishment and maintenance of close social contacts [44,55–58]. Rats use low-frequency (22-kHz) alarm calls as a warning to conspecifics of imminent dangers, with a view to promoting a survival-enhancing behavioural strategy in the colony as a whole [59–61]. 22-kHz USVs can be elicited by pharmacological stimulation of the lateral PAG [62], but also by electrical stimulation of the dorsal (dorsomedial + dorsolateral) and even ventrolateral PAG [63].

The purpose of the present study was to ascertain whether neurons of the parvafox nucleus play a role in the expression of positive emotions in rats, as evidenced by the production of 50-kHz USVs. To this end, the tickling-induced vocalisations that were emitted by adolescent Wistar rats, as well as the tendency to seek (approach and follow) the experimenter’s hand, were analysed before and

after lesioning of the LHA in the region of the parvafox nucleus by targeted injections of the excitotoxin kainic acid.

2. Materials and methods

2.1. Animals

The experiments were performed on three cohorts of animals. In each cohort, Wistar-rat pups of a common litter and of both sexes were weaned after three weeks of age ($n = 10$ in cohort one, $n = 11$ in cohort two, $n = 16$ in cohort three). They were housed individually in cages ($42.5 \times 21 \times 20$ cm) containing bedding and a cardboard tube, for up to four weeks. The rats had *ad libitum* access to water and to rodent chow, and were maintained on a 12-h-light/12-h-dark cycle. All procedures were conducted in strict accordance with the Swiss laws on animal experimentation and were approved by the Veterinary Commission for Animal Research of the Canton of Fribourg.

2.2. Tickling experiment

In all three cohorts, rats underwent a tickling session on Mondays, Tuesdays and Wednesdays during a four-week period (12 sessions in all). After a baseline period spanning the first two weeks of tickling, surgery was performed. The experimental design is summarized in Fig. 1. Each tickling session (duration = 5 min per rat) was comprised of ten 30-s manipulations (positions), which included handling and break-periods. The manipulations followed the protocol that was elaborated by Schwarting et al. [64] and were performed in a cage ($54.5 \times 37 \times 20$ cm) without bedding. The very first tickling session per cohort involved four break-periods at positions 1, 3, 5 and 8. In the 11 subsequent tickling sessions, the number of break-periods was reduced to two, at positions 2 and 5. Furthermore, the very first tickling session per cohort included two standardised handling procedures (stroking the rat from head to tail at position 2, and passing it from one hand to the other at position 4), which were reduced to one (passing the rat from one hand to the other at position 1) in the 11 subsequent tickling sessions. In the remaining positions, the tickling manipulations that were referred to as “push & drill” (x2), “grab & tickle” (x2), “hand-chase” (x1), “neck tickle” (x1) and “belly tickle” (x1) in the original publication [64] were performed. In the very first tickling session per cohort, only the first four of the manipulations were performed once. In each cohort, all tickling sessions were executed by the same experimenter, who was blind to the treatment allocations and who was not involved in any stage of the experimental analysis. Only rats that emitted sufficient numbers of 50-kHz USVs at baseline (in general: ≥ 100 USVs in total during the 10×30 s of tickling manipulations) were used for surgery and for the post-surgical tickling sessions (cohort 1: $n = 5$, cohort 2: $n = 7$, cohort 3: $n = 5$).

2.3. Surgery and lesioning

Rats were anaesthetised by injecting a saline (0.9%) mixture of ketamine (60 mg/kg of body weight) and xylazine (15 mg/kg of body weight) into the peritoneal cavity. The head of the animal was secured in a stereotaxic apparatus (Kopff) and craniotomy was performed over the targeted region in the lateral hypothalamus on both sides. Excitotoxic lesioning of the parvafox nucleus was induced by a local injection of kainic acid [Sigma (1% in saline)]. The acid was injected into the test animals (cohort 1: $n = 3$, cohort 2: $n = 5$, cohort 3: $n = 4$) via a fine-bored needle (external diameter: 0.14 mm; Ga: 34) which was connected to a 2.5- μ l Hamilton syringe that was mounted on a manual microinjection unit (Kopff, modell 5000). Two injections, separated by a rostro-caudal distance of 0.7 mm, were delivered on each side (4 in all) at the following stereotaxic coordinates (in mm, with respect to Bregma and to the brain surface): antero-posterior (AP): -2.3 and -3 ; medio-lateral (ML): ± 2.2 ; dorso-ventral (DV): -9 . At each site, 55–75 nl of the excitotoxin were injected over a period of 1 min. After the injection, the needle was left in place for 3–5 min to allow for drug diffusion. The needle was then withdrawn, the skin over the skull was sutured, and the animals were left to recover. In the control animals, we either injected a solution of physiological saline (0.9%) instead of the kainic acid, using the above-described protocol (saline controls; cohort 1: $n = 1$, cohort 2: $n = 1$), or merely cut and sutured the skin over the skull (suture controls; cohort 1: $n = 1$, cohort 2: $n = 1$, cohort 3: $n = 1$). The mean body weight of the rats on the day of surgery was 141.3 g in cohort 1, 137.6 g in cohort 2 and 188.6 g in cohort 3.

2.4. Recording and analysis of ultrasonic vocalisations (USVs)

During all tickling sessions, ultrasonic vocalisations (USVs) were detected by an ultrasonic condenser microphone (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany), which was located roughly 22 centimetres above the cage floor and laterally extended into the cage by about 3 centimetres. The acoustic signals were amplified and then digitized at 300 kHz with 16-bit resolution (UltraSoundGate 116H, Avisoft Bioacoustics) and were displayed on a computer screen in real-time using Recorder.USGH software (Avisoft Bioacoustics). USVs were analysed off-line using SASLab Pro 5.1 software (Avisoft Bioacoustics). Spectrograms were produced using the following parameters: FFT-length = 512, frame-size = 75%, window: hamming, overlap = 50%. A cut-off frequency of 18 kHz was set to eliminate insignificant low-frequency noise, and individual USV-elements were manually selected. The total number of USVs that were emitted during the 5 min of tickling was determined as well as duration and peak frequency of individual calls, which served as a basis for the classification into the three different categories that have been described by Schwarting et al. [64]: high-frequency calls (peak frequency > 33 kHz: “50-kHz calls”), long low-frequency calls (peak frequency < 33 kHz, USV-duration > 300 ms: “22-kHz

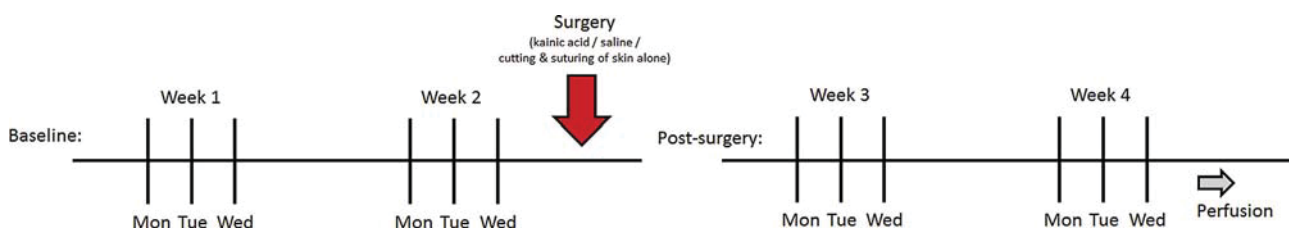


Fig. 1. Schematic representation of the experimental design.

Rats were tickled on Mondays, Tuesdays and Wednesdays for a period of four weeks. At the end of the second week, surgery was performed (test animals: stereotactic injection of kainic acid into the parvafox nucleus region; control animals: analogous injection of saline or mere cutting and suturing of the skin). Each of the vertical lines represents a 5-min period of tickling and a performance of the “hand-approach latency” (HAL) test.

calls”) and short low-frequency calls (peak frequency <33 kHz, USV-duration <300 ms).

2.5. Recording and analysis of behaviour

A regular video camera, placed above the cage, was used to record the behaviour of each rat during the tickling sessions and during the “hand- approach latency” (HAL) test which was always conducted immediately afterwards [53].

In the HAL-test, the rat was placed in one corner of the same cage that was used for tickling, facing the back of the experimenter’s hand, which was placed in the corner diagonal to the rat, and the latency to approach the hand was recorded. Whenever a rat touched the hand with its head or whiskers, it received 10 s of “grab & tickle” stimulation before being put back into the same corner. It was permitted to approach the experimenter’s hand again for up to a total of five times. If a rat did not touch the experimenter’s hand within 30 s, the HAL-test was terminated and the rat was put back into its home cage. The following two parameters were quantified in an off-line analysis of the HAL test videos: the total number of hand approaches (0–5), and, for each successful approach, the latency to touch the experimenter’s hand (0–30 s). These two parameters were combined to yield an “HAL-positivity index”, which was derived by dividing the total number of hand approaches by the mean latency to touch the experimenter’s hand.

An off-line analysis of the video-taped tickling sessions was conducted to determine the total time during which individual rats actively followed the experimenter’s hand during the “hand-chase” manipulation. Observer XT 11 software was used to estimate the total following time.

2.6. Immunohistochemistry

Rats were perfused at the end of the fourth week with physiological saline, followed by a 4% paraformaldehyde solution. Details of the procedure are described elsewhere [6]. 80- μ m-thick coronal cryosections through the brain specimens were collected in 0.1 M phosphate buffer (pH 7.3). All sections along the rostrocaudal aspect of the parvafox nucleus (in general 28 [range: 25–30] sections, spanning a total length of 2.24 [range: 2.0–2.4] mm) were incubated first with a monoclonal primary antibody against PV (PV235, 1:1000, Swant, Marly, Switzerland) for 24–48 h at 4 °C, then with biotinylated anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA) for 2 h at ambient temperature, and finally with streptavidin-Alexa488 (1:200, Jackson Immunoresearch Laboratory, West Grove, PA) for 3 h at ambient temperature. In addition, every fourth section of the specimens from rats in cohort two was exposed first to rabbit anti-Iba1 (ionized calcium-binding adaptor molecule 1; Wako Pure Chemicals, Osaka, Japan [0.25 μ g/ml]) for 24 h at 4 °C and then to Cy3-conjugated donkey anti-rabbit antibody (Jackson Immunoresearch Laboratory [1:200]) for 2 h at ambient temperature.

2.7. Image analysis

In each section along the rostrocaudal aspect of the parvafox nucleus, PV-immunoreactive cells located in the nucleus region were counted in a LEICA DM6000 epifluorescence microscope connected to a C4742-95-12NR camera (Hamamatsu Photonics, Hamamatsu, Japan), using a \times 20-objective.

2.8. Statistical analysis

IBM SPSS Statistics 21.0 was used for all statistical analyses and to create graphs.

One-way, repeated-measures ANOVA was used to investigate baseline changes in the three parameters of interest (high-frequency vocalisations, HAL-positivity index, following time) on a group basis. Since Mauchly’s test of sphericity was not statistically significant, sphericity was assumed.

Box-plots display data appertaining to each group, whereas regression analyses compare the data of individual animals.

For regression analyses, the following regression models were applied: linear, logarithmic, power, logistic, exponential. The model with the lowest *p*-value was selected for demonstration.

Statistical significance was set at *p* < 0.05.

3. Results

3.1. Evaluation of lesions

3.1.1. Semi-quantitative analysis of parvalbumin-immunoreactive (PV-ir) cells in the parvafox nucleus region

Control animals in the three cohorts (*n*=5) did not have comparable numbers of PV-ir cells (cohort 1: 164 \pm 23 cells per side [mean \pm SD]; cohort 2: 110 \pm 18; cohort 3: 321 \pm 26). Hence, in the lesioned test animals, the total number of PV-ir cells per side was expressed as a percentage of the mean total number on the same side in the (1–2) control animal(s) of the same cohort (Table 1). Since the longitudinally extending PV core of the parvafox nucleus spans a width of no more than about 0.5 mm in the dorsoventral and the mediolateral planes, difficulty was experienced in hitting it precisely with the stereotactic injection needle. Consequently, the percentages of the total numbers of PV-ir cells in the kainic acid injected test animals varied widely, namely between 7% and 155%. Test animals with a percentage of <60% on both sides were classified as having bilaterally successful lesions (*n*=5); those with a percentage of <60% on one side, but >60% on the other were classified as having unilaterally successful lesions (*n*=4); and those with a percentage of >60% on both sides as having control-like lesions (*n*=3). Cell-counting data appertaining to individual sections through the parvafox nucleus of rats in each of the four groups are depicted in Fig. 2; those appertaining to each of the 17 individual rats, as well as positions of the injection needles, are presented in Supplementary Fig. 1.

3.2. Staining for microglia

Immunostaining with antibodies against Iba1 was performed to visualise the microglia on sections through the brains of the seven animals in cohort two. Iba1 is a calcium-binding protein specifically expressed in microglia [65], which are activated in regions where cell death and inflammation occur (see, for example, [66]). Microglia were considered to be activated if they were present at high density and if they manifested an enlarged soma with relatively few cytoplasmic processes. No activated microglia were observed in the brain of the suture-control rat (Fig. 3A). In the saline-control rat, activated microglia were encountered along some of the four needle-tracks (not shown). In rats classified as having control-like lesions, activated microglia were likewise detected along needle-tracks or in accumulations close by, but these accumulations were usually located either dorsally or laterally relative to the parvafox nucleus. The sole exception was rat 381.12, in which some activated microglia were detected within the core of the nucleus, amongst a normal number of PV-ir cells, as well as within the surrounding shell of Foxb1-expressing cells, which could not be visualised in the present study. Most importantly, in both rats classified as having unilaterally successful lesions, dense accu-

Table 1

Quantification of PV-ir cell numbers and consequent group assignment of the 17 rats used in the study.

Cohort	Rat	Left side Absolute numbers of PV-ir cells counted	Right side Absolute numbers of PV-ir cells counted	Left side Numbers of PV-ir cells counted, expressed as a percentage of the respective cohort-control values	Right side Numbers of PV-ir cells counted, expressed as a percentage of the respective cohort-control values	Group
1	141/12	189	181	112%	113%	Suture-control
1	142/12	157	130	88%	87%	Saline-control
1	140/12	32	43	19%	35%	Bilaterally successful lesion
1	143/12	63	65	40%	53%	Bilaterally successful lesion
1	144/12	42	42	22%	28%	Bilaterally successful lesion
2	382/12	97	140	90%	109%	Suture-control
2	379/12	96	105	110%	91%	Saline-control
2	381/12	119	102	124%	101%	Control-like lesion
2	384/12	108	120	117%	121%	Control-like lesion
2	385/12	149	170	155%	136%	Control-like lesion
2	380/12	80	34	106%	44%	Unilaterally successful lesion
2	383/12	53	124	55%	124%	Unilaterally successful lesion
3	68/15	347	295	100%	100%	Suture-control
3	69/15	185	314	53%	106%	Unilaterally successful lesion
3	73/15	57	206	16%	70%	Unilaterally successful lesion
3	71/15	127	20	37%	7%	Bilaterally successful lesion
3	74/15	64	120	18%	41%	Bilaterally successful lesion

Absolute and relative numbers of PV-ir cells counted on both sides in individual rats. The relative numbers represent percentages of control-rat values. Since the control animals in the different cohorts did not have comparable numbers of PV-ir cells, lesioning efficiency was always assessed by comparing cell numbers for animals in the same cohort only. 60% was defined as the threshold value for a successfully lesioned side.

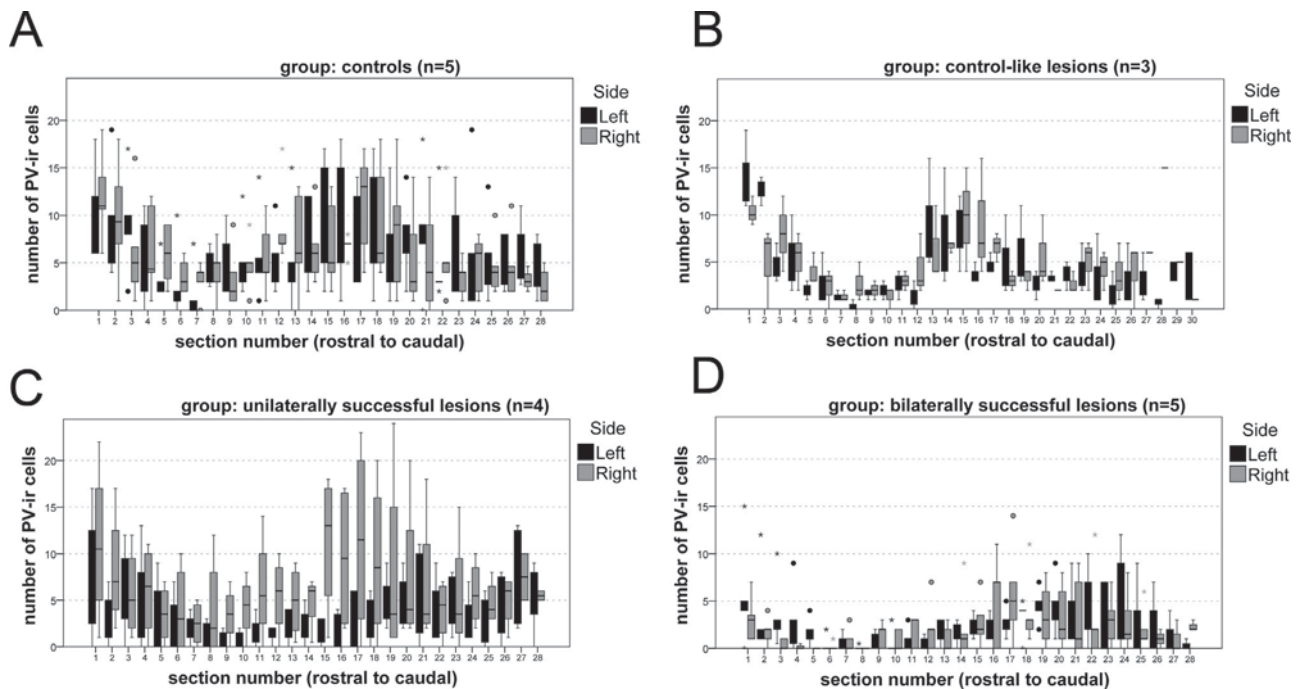


Fig. 2. Classification of rats into four groups according to the number of parvalbumin-immunoreactive (PV-ir) neurons that survived excitotoxic lesioning of the paravox nucleus region with kainic acid.

Numbers of PV-ir cells counted on individual sections through the paravox nucleus in the four groups of rats. (A): controls ($n=5$); (B): control-like lesions ($n=3$); (C): unilaterally successful lesions ($n=4$); and (D): bilaterally successful lesions ($n=5$). Data appertaining to the left and the right paravox nucleus are depicted in black and grey, respectively. In these and in all subsequent box-plots, outliers (values at a distance of 1.5–3.0 box-lengths from the box) are indicated with circles and extreme values (at a distance of >3 box-lengths from the box) with asterisks. Note the typical distribution of the PV-ir cells along the rostrocaudal axis in the control rats (A), and the clear reduction in cell numbers at most of the rostrocaudal levels in the animals with bilaterally successful lesions (D).

mulations of activated microglia, centred around the PV-ir cells or their dendritic remnants, were revealed in several of the sections through the successfully lesioned side (Fig. 3B).

3.3. Evaluation of ultrasonic vocalisations (USVs)

The absolute numbers of USVs in the three categories (high-frequency or 50-kHz calls/short low-frequency calls/long low-

frequency or 22-kHz calls), that were emitted by rats in the four groups during the 12 tickling sessions are represented in Fig. 4.

The number of high-frequency calls increased significantly during the six baseline days in the group of all 17 rats used in this study (ANOVA, $F=19.23$, $p<0.001$). When the four rat groups were analysed separately, the finding was the same in each group except that in which control-like lesions had been produced. In this latter group, the parameter had plateaued by about tickling day 4. With

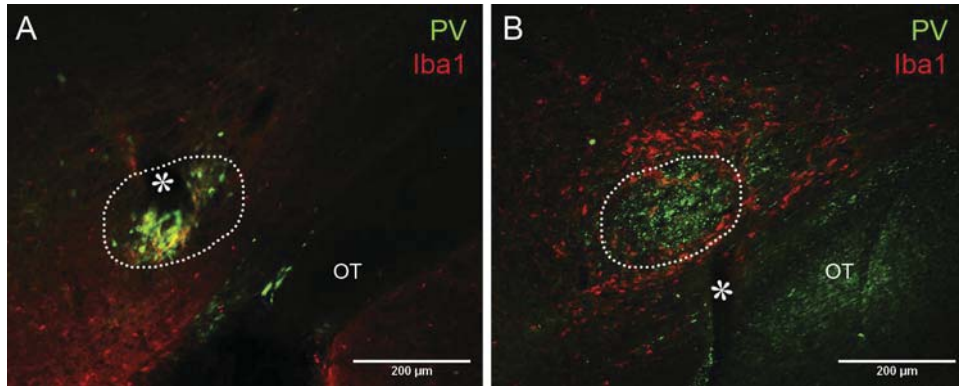


Fig. 3. Activated microglia appear in regions where neuronal death is evident.

Fluorescence photomicrographs of two exemplary sections after immunostaining for PV (green) and for a microglial marker (Iba1, red) in a suture-control rat (A) and in a rat with a unilaterally successful lesion (B). Activated microglia are absent from the brain of the suture-control rat (A), as evidenced by the relatively low numerical density of the cells, their small somal size and their abundant cytoplasmic processes. Microglia were encountered along needle-tracks, even after an injection of saline. After an injection of kainic acid, they were observed close to the tip of the needle. Dense accumulations of microglia, with morphological features suggestive of an activated state (enlarged somal size and relatively few cytoplasmic processes), were detected in the area of the parvafox nucleus (encircled with a broken line) only in conjunction with successful lesioning (B). White asterisks point to arterioles, which are typically observed in the parvafox nucleus region. OT = optic tract.

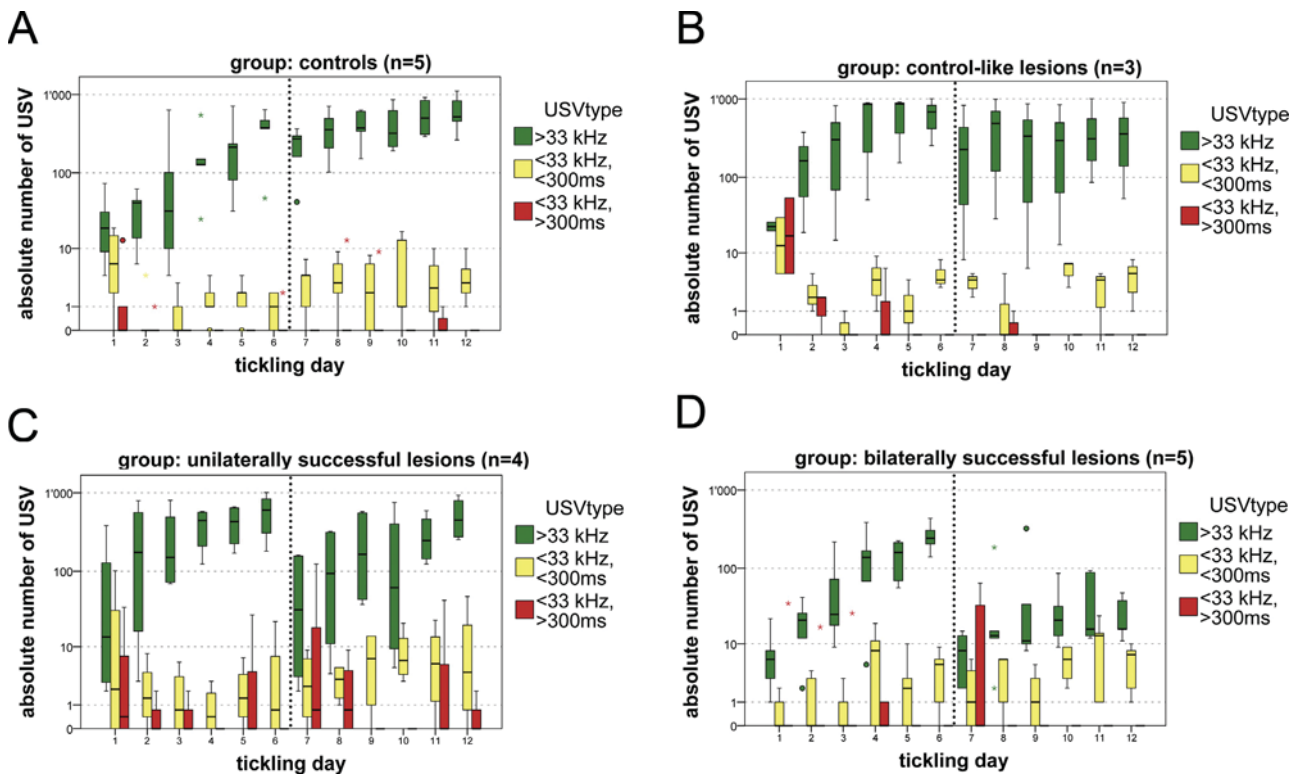


Fig. 4. Numbers of high-frequency USVs (“50-kHz calls”, >33 kHz) are greatly reduced after bilaterally successful lesioning of the parvafox nucleus (PV sub-population).

Absolute numbers of ultrasonic vocalisations (USV) in the three categories that were emitted by the four groups of rats during individual tickling sessions. Green boxes correspond to high-frequency calls (“50-kHz calls”: peak frequency >33 kHz), yellow boxes to short low-frequency calls (peak frequency <33 kHz, duration <300 ms), and red boxes to long low-frequency calls (“22-kHz calls”: peak frequency <33 kHz, duration >300 ms). In this figure and in subsequent ones, broken vertical lines separate baseline from post-surgical tickling sessions. In control rats, the number of 50-kHz calls emitted after surgery was comparable to or even higher than that on baseline day 6 (A), whereas in rats with bilaterally successful lesions, it was greatly reduced (D). After surgery, 22-kHz calls were produced predominantly by rats with uni- (C) or bilaterally (D) successful lesions. Note that the ordinate scales are logarithmic.

the exception of one single animal (control 141_12), each of the rats produced more high-frequency calls at later than at earlier baseline days.

For each rat, the number of high-frequency calls emitted during each tickling session was expressed as a percentage of the number of high-frequency calls emitted during the last baseline session (tickling day 6, set to 100%). Fig. 5 depicts relative numbers of high-frequency calls emitted by rats that were classified together

into one of the four groups. In Supplementary Fig. 2 (right parts: S2A2–S2R2) the same data are presented for each rat individually. In all four groups, the number of high-frequency calls decreased between the last baseline day (tickling day 6) and the first post-surgical day (tickling day 7) in at least one animal. However, in control animals (Fig. 5A) and in animals with control-like lesions (Fig. 5B), the number of high-frequency calls had returned to or had overshot baseline levels by tickling day 8. The sole exception

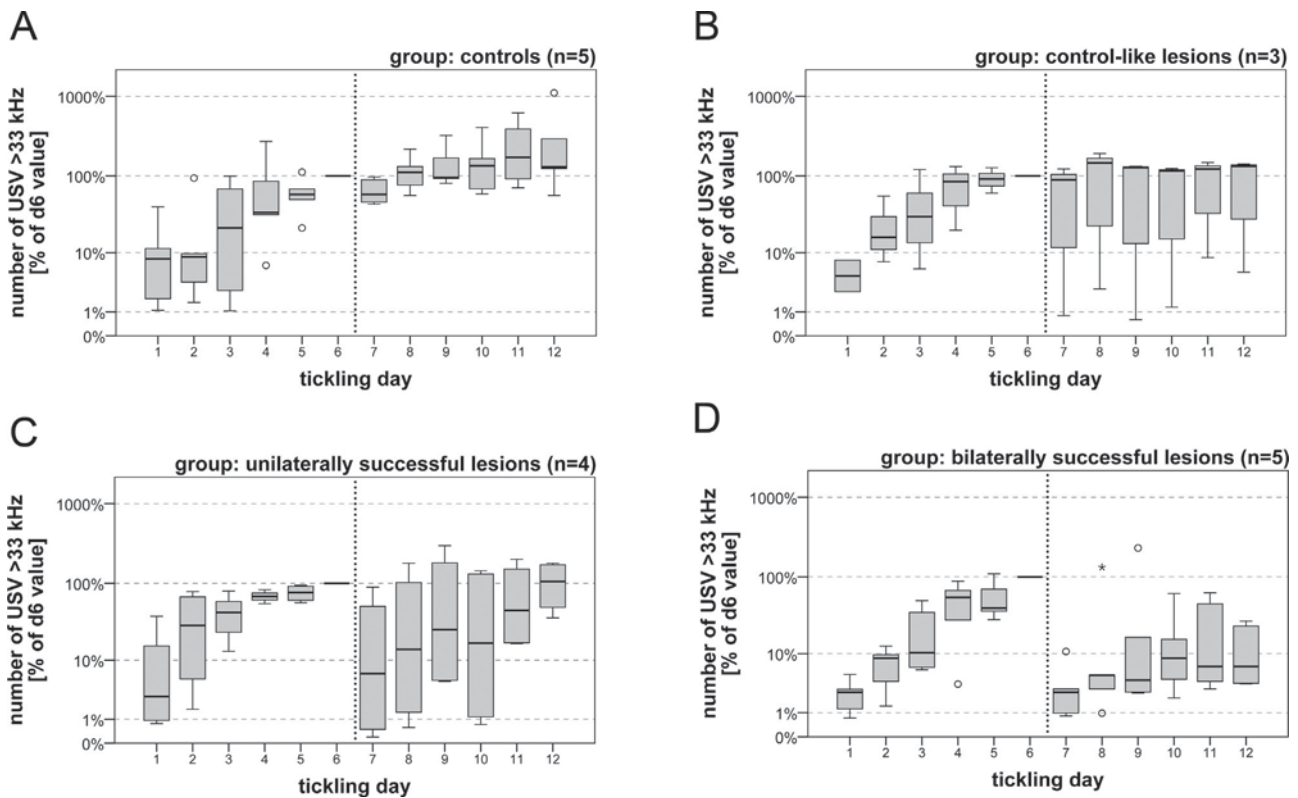


Fig. 5. Numbers of high-frequency calls are reduced more profoundly and more persistently after bilaterally than after unilaterally successful lesioning of the parvafox nucleus (PV sub-population).

Relative numbers of high-frequency calls (“50-kHz calls”: peak frequency >33 kHz) emitted by the four groups of rats during individual tickling sessions, expressed as a percentage of the number of high-frequency calls emitted by each rat during the last baseline session (day 6, set to 100%). In the control group, the mean relative number of 50-kHz calls that were emitted post-surgically varied between 67% (day 7) and 341% (day 12) of the day-6 baseline values (A), whereas in rats with bilaterally successful lesions, it was reduced to between 4% (day 7) and 51% (day 9, note the extreme outlier) of the day-6 baseline values (D). Note that the ordinate scales are logarithmic.

was rat 381_12, in which the number of high-frequency calls was markedly reduced during the six post-surgical sessions throughout (Fig. S2F). Most importantly, in the group of animals with bilaterally successful lesions, the number of high-frequency calls was greatly reduced after lesioning, at least during the first four tickling sessions (Fig. 5D), with only one exception (74_15, see Fig. S2R). In rats with unilaterally successful lesions (Fig. 5C), the situation resembled that in either the control animals (69_15, Fig. S2L and 73_15, Fig. S2M) or in the rats with bilaterally successful lesions (380_12, Fig. S2I and 383_12, Fig. S2K).

The number of vocalisations in each of the three USV categories that were produced by individual rats during the 12 tickling sessions are shown in Supplementary Fig. 2 (left parts: S2A1-S2R1). Long low-frequency calls were emitted mainly during the first baseline week. During the last two baseline tickling sessions, they were produced by only two rats (141_12, Fig. S2A1 and 380_12, Fig. S2I1). After surgery, long low-frequency calls were emitted mainly by several of the rats classified as having uni- or bilaterally successful lesions (Fig. S2I1-S2R1), but also by one in the saline-control group (379_12, Fig. S2D1).

Of all the USVs that were emitted by rats during the first four tickling sessions after the production of bilaterally successful lesions, 9.9% were long low-frequency calls and only 83.1% high-frequency ones (Table 2). In the group of rats with unilaterally successful lesions, 4.0% of the USVs were long low-frequency calls and 93.2% high-frequency ones. In the other two groups of animals, at least 98.6% of all USVs emitted post-surgically by the tickled rats were high-frequency calls; no more than 0.3% of the USVs were long low-frequency ones.

We conclude from these data that successful lesioning of PV-ir neurons in the parvafox nucleus is accompanied by a reduction in the number of 50-kHz calls and, in some cases, by an increase in the number of 22-kHz ones. Furthermore, unilaterally successful lesioning on either the left or the right side likewise had the capacity to elicit a reduction in the number of 50-kHz calls.

3.4. Evaluation of behavioural data

The inter-individual performances in the “hand-approach latency (HAL) test” (HAL-positivity index) varied greatly, even under the same conditions (e.g. between the suture-controls at baseline). Furthermore, also the intra-individual HAL-performance of several of the rats between the experimental days was subjected to a considerable variation.

The HAL-positivity index increased significantly during the six baseline days in the group of all 17 rats used in the present study (ANOVA, $F=5.42$, $p<0.001$). However, when the four rat groups were analysed separately, this finding held true only for the group of animals with control-like lesions. Moreover, a few animals attained higher values during the first than during the second baseline week. For these reasons, all six baseline values for the HAL-positivity index were compared to all six post-surgical ones.

The mean HAL-positivity index for each of the four rat groups during the baseline days varied between 0.159 and 1.112 (Table 2). In each of the four groups, the mean HAL-positivity index was higher at baseline than after surgery. The value decreased to 78% of the baseline mean in the control group (0.253 vs. 0.197), to 68% in animals with control-like lesions (1.112 vs. 0.751), to 71% in

Table 2

Group-wise comparison of numbers of emitted vocalisations per call-type, of HAL-positivity index, and of hand-following time before and after surgery.

Group-condition	High-frequency calls: mean (row sum%)	Short low-frequency calls: mean (row sum%)	Long low-frequency calls: mean (row sum%)	HAL-positivity index	HAL-positivity index	Hand-following time	Hand-following time
				[/s]: Mean \pm SD	[/s]: Range	[% of day-6 value]: Mean \pm SD	[% of day-6 value]: Range
Controls-baseline	277 (99.4%)	1 (0.5%)	0 (0.0%)	0.253 \pm 0.203	0.062 – 0.630	88.6 \pm 29.5	42.2 – 121.5
Controls-postsurgery	367 (98.6%)	4 (1.1%)	1 (0.3%)	0.197 \pm 0.087	0.108 – 0.338	67.7 \pm 34.3	23.7 – 125.0
Control-like lesions-baseline	631 (99.3%)	4 (0.6%)	1 (0.1%)	1.112 \pm 0.422	0.650 – 1.671	125.9 \pm 19.1	99.2 – 143.0
Control-like lesions-postsurgery	411 (99.3%)	3 (0.7%)	0 (0.0%)	0.751 \pm 0.603	0.049 – 1.522	62.9 \pm 29.8	29.0 – 101.5
Unilaterally successful lesions-baseline	481 (98.9%)	3 (0.7%)	2 (0.5%)	0.484 \pm 0.264	0.211 – 0.814	86.7 \pm 20.0	62.8 – 111.8
Unilaterally successful lesions-postsurgery	198 (93.2%)	6 (2.8%)	9 (4.0%)	0.344 \pm 0.181	0.167 – 0.595	49.9 \pm 14.8	29.4 – 68.2
Bilaterally successful lesions-baseline	188 (97.4%)	5 (2.6%)	0 (0.1%)	0.159 \pm 0.152	0.024 – 0.450	97.0 \pm 9.4	87.1 – 108.4
Bilaterally successful lesions-postsurgery	41 (83.1%)	3 (6.9%)	5 (9.9%)	0.088 \pm 0.078	0.021 – 0.228	32.0 \pm 21.8	8.5 – 73.1

For vocalisations as well as for hand-following time, data gleaned from tickling days 4–6 were included in the baseline condition, and those from tickling days 7–10 in the post-surgical one. However, for the HAL-positivity index, data gleaned from all six available tickling sessions were included in the baseline as well as in the post-surgical condition. See Section 3 for an explanation of the rationale behind this approach.

those with unilaterally successful lesions (0.484 vs. 0.344), and the strongest reduction – to 55% – was observed in rats with bilaterally successful lesions (0.159 vs. 0.088).

Data appertaining to the HAL-positivity index on individual experimental days are depicted in Fig. 6A–D. These graphs reveal that for animals in each of the four groups, there was at least one day after surgery on which they approached the experimenter's hand much less than on the last baseline day (tickling day 6). In all groups, including the controls, a pronounced reduction in the mean HAL-positivity index was observed between the last baseline and the first post-surgical day (tickling day 7).

The performance of individual rats in actively following the experimenter's hand during the "hand-chase" manipulation of the tickling sessions varied considerably, too. Similarly to the HAL-positivity index, the total hand-following time increased significantly during the six baseline days in the group of all 17 rats used in the present study (ANOVA, $F=2.52$, $p=0.036$). The same finding held true when the four rat groups were analysed separately, for the groups of the controls as well as animals with bilaterally successful lesions. For all 17 rats, the total hand-following time was larger at later than at earlier baseline days. As for the high-frequency vocalisations, the total following time on the last three baseline days (tickling days 4–6) was thus compared to that on the first four post-surgical days (tickling days 7–10).

In absolute values, the mean total hand-following time varied between 14.87 and 17.96 s for the four rat groups on the last three baseline days (data not shown). Only one rat in the control group did not follow the experimenter's hand at all during one of baseline days 4–6 (379.12 on baseline day 4). For each rat, the total hand-following time on baseline day 6 (17.83 ± 4.69 s; range: 11.74–28.89 s) was set to 100%; the hand-following times on the last three baseline days, as well as on the first four days after surgery, were expressed as percentages of this value. The mean relative hand-following times varied between 86.7% and 125.9% for the four rat groups on the last three baseline days (Table 2). Overall, the animals in each of the four groups followed the experimenter's hand less well on the first four days after surgery than on the last baseline day (relative following times <100%). Moreover, in all four groups, the mean hand-following time on the first four days after surgery was lower than the mean following time on the last three baseline days. The most pronounced reduction in the mean relative following time was observed in the group of animals with bilaterally successful lesions. The value decreased from 97.0% before lesioning to 32.0% thereafter, which corresponds to a reduction to 33% of late baseline values. In the other three groups, the mean relative following time was reduced only to 58% (unilaterally

successful lesions), 50% (control-like lesions) and 76% (controls) of the late baseline values.

Data appertaining to the relative hand-following times on individual experimental days are presented in Fig. 7A–D. These graphs reveal that in controls as well as in the animals with control-like lesions, the relative hand-following time, although somewhat reduced, remained relatively constant during the post-surgical days. In the group of rats with unilaterally successful lesions, the relative hand-following time decreased markedly between the last baseline and the first post-surgical day; during the subsequent five post-surgical days, the values were similar to those in the controls. Only in the group of animals with bilaterally successful lesions was the reduction in the relative hand-following time between the last baseline and the first post-surgical day protracted thereafter.

We conclude from these data that successful lesioning of PV-ir neurons in the parvafox nucleus is accompanied by a reduction in the rat's tendency to follow the experimenter's hand, which exceeded that accompanying the surgical manipulations that spared the resident population of PV-ir neurons.

Supplementary video 1 (electronic version only) depicts the behaviour of a suture control rat (382.12) and of a rat with a unilaterally successful lesion (383.12) on the second post-surgical day (tickling day 8) during two exemplary tickling manipulations ("neck tickle" and "push & drill"), as well as during the following of and the approach towards the experimenter's hand during the "hand-chase" manipulation and the HAL-test, respectively.

3.5. Regression analyses/curve estimations

To tackle our main question, namely, whether successful lesioning of the parvafox nucleus leads to a reduction in the number of high-frequency calls, the total number of PV-ir cells was related to the level to which the total number of high-frequency calls was reduced during the first four post-surgical days (days 7–10) compared to the call levels that were attained during the last three baseline days (days 4–6) in each of the 17 rats. The curve that was found to best describe the relationship between these two parameters was a power function ($R=0.65$, $p=0.005$; $y=0.0004x^{2.16}$) (Fig. 8A).

We asked next whether the number of PV-ir cells correlated with surgery-associated changes in the other two parameters that were used to assess positive affective behaviour: the propensity to follow (hand-chase: following time) or approach (HAL-test: HAL-positivity index) the experimenter's hand. Also the relationship between the surgery-associated change in following time and the total number of PV-ir cells was best fitted by a power function ($R=0.59$, $p=0.012$; $y=1.47x^{0.65}$) (Fig. 8B). On the other hand,

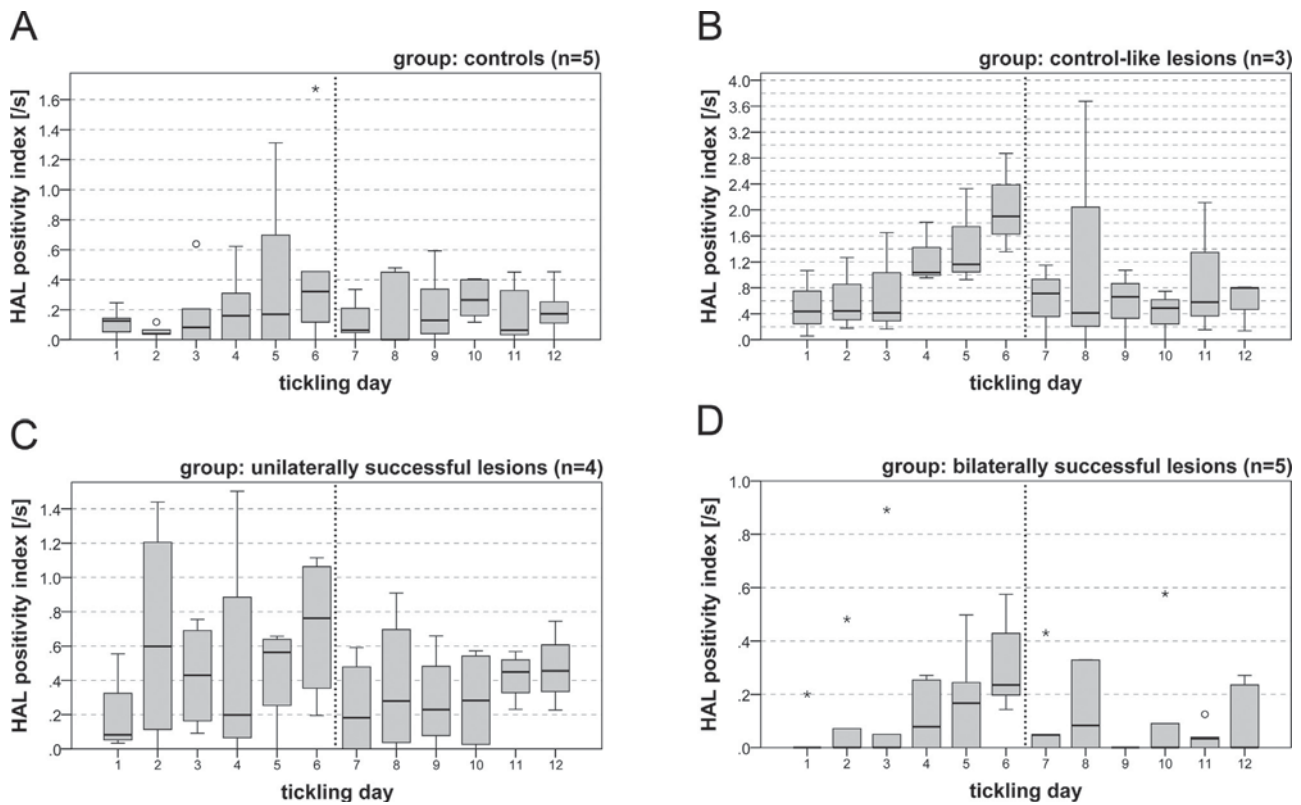


Fig. 6. The most profound reduction in the HAL-positivity index (HAL-test) was observed in rats with bilaterally successful lesions. The HAL-positivity index for each rat in the four groups was derived from the results of the hand-approach latency (HAL) test conducted after each tickling session. The HAL-positivity index was derived by dividing the total number of hand approaches by the mean latency to touch it. In the control group, the mean HAL-positivity index on post-surgical days varied between 26% (day 7) and 53% (day 10) of the day-6 baseline values (A), whereas in rats with bilaterally successful lesions, it varied between 0% (day 9) and 47% (day 8) of the day-6 baseline values (D). Note that the ordinate scales span different ranges.

the relationship between the surgery-associated change in the HAL-positivity index and the total number of PV-ir cells was best described by a linear regression ($R=0.53, p=0.028; y=0.33x+5.49$) (Fig. 8C). Taken together, the absolute numbers of PV-ir cells counted in the 17 rats correlated with each of the three parameters used in the present study to quantify positive affect-related behaviour, whilst the characteristics of the relationships varied from parameter to parameter.

Further regression analyses were undertaken to probe the nature of the relationship between surgery-associated changes in the three different parameters used to assess positive affective behaviour. The relationship between the surgery-associated changes in the number of high-frequency calls and in the hand-following time was again best fitted by a power function ($R=0.64, p=0.006; y=20.97x^{0.21}$) (Fig. 8D). The relationship between the surgery-associated changes in the number of high-frequency calls and in the HAL-positivity index was best described by a logarithmic function ($R=0.59, p=0.013; y=-19.63+28.10 \times \log[x]$) (Fig. 8E). Of all the correlations, the linear relationship between the surgery-associated changes in the hand-following time and in the HAL-positivity index was the closest ($R=0.76, p<0.001; y=2.27x-35.97$) (Fig. 8F). In other words, rats in which the number of high-frequency vocalisations after surgery was reduced relative to baseline, also followed the experimenter's hand less well and approached it less than during the baseline sessions. The correlation between the two parameters that related to the experimenter's hand was stronger than that existing between either of these and the high-frequency vocalisations.

4. Discussion

With a view to ascertaining whether parvalbumin-immunoreactive (PV-ir) neurons in the paravox nucleus play a role in the vocalisation and positive affective behaviour of rats, we adopted an approach that combined tickling with excitotoxic lesioning of the targeted lateral hypothalamic structure. Earlier studies on the effect of lesions on vocalisations and related emotional behaviours have been conducted mainly on primates or on mammalian species other than the rat [67]. We are aware of only one such study in rats, which demonstrated a reduction in frequency-modulated 50-kHz calls after a destruction of the mesolimbic dopamine system [22].

In preliminary experiments, we attempted to lesion the paravox nucleus using electrolytic lesions or a stereotactic injection of ibotenic acid. In the former case, the lesions were highly unspecific and implicated also the nerve-fibre tracks, whereas in the latter, the damage was very limited, with maximally 20–30% of the PV-ir cells being eliminated. In our hands, a cerebral injection of kainic acid proved to be the most efficacious and useful method of lesioning.

Of the four groups of rats that were established (controls, control-like lesions, unilaterally and bilaterally successful lesions), the one in which the animals had bilaterally successful lesions manifested the most profound reduction in the 50-kHz call-numbers on the post-surgical days relative to the baseline levels (Figs. 4 and 5). In two of the animals with unilaterally successful lesions, considerable reductions in the number of 50-kHz calls were likewise observed during the first few post-surgical days (Fig. S2). But in these two cases (380.12 and 383.12), the parameter rose again steeply during the very last two sessions, which could reflect a compensatory response of the neurons on the better preserved

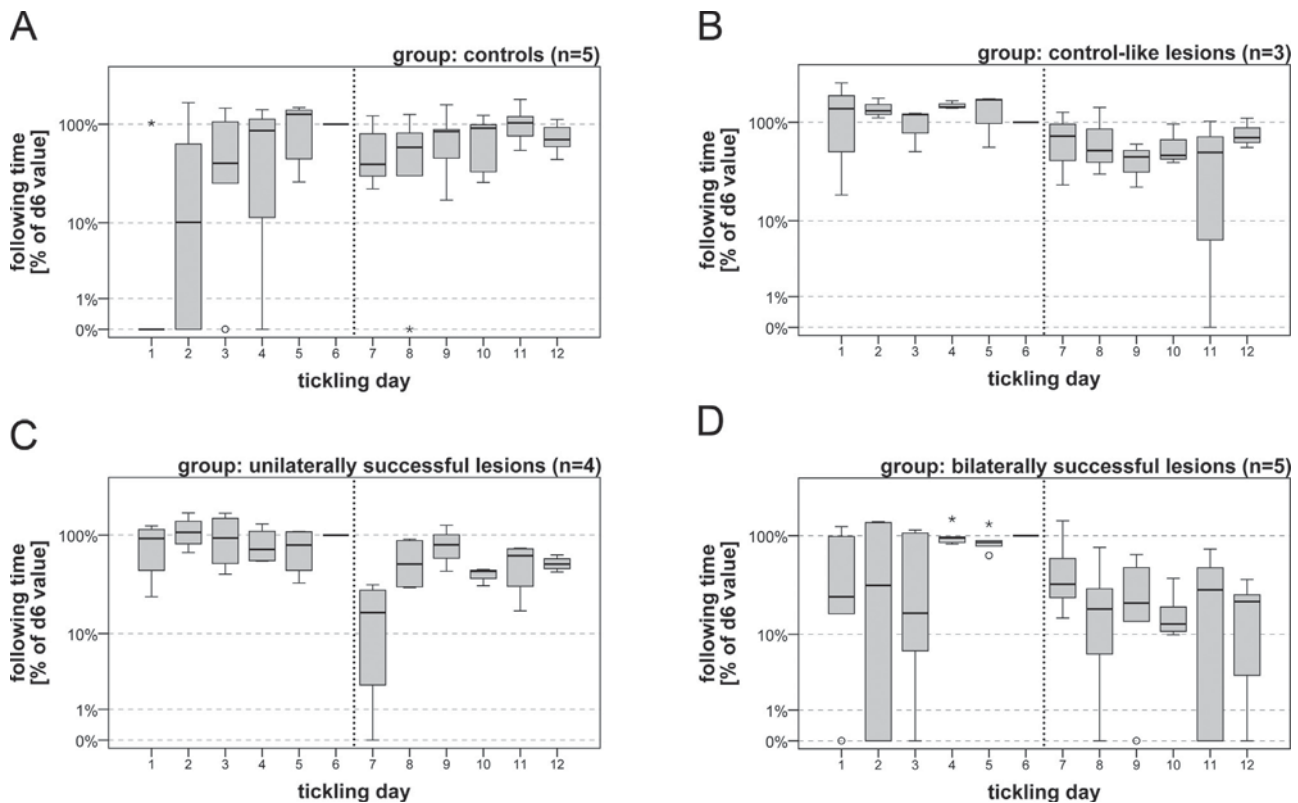


Fig. 7. The greatest and most persistent reduction in the hand-following time was observed in rats with bilaterally successful lesions. Relative hand-following times of rats in the four groups during the “hand-chase” manipulation of individual tickling sessions, expressed as a percentage of the following time for each rat during the last baseline session. In the control group, the mean following time on post-surgical days varied between 59% (day 7) and 106% (day 11) of the day-6 baseline values (A), whereas in rats with bilaterally successful lesions it varied between 18% (day 12) and 54% (day 7) of the day-6 baseline values (D). Note that the ordinate scales are logarithmic.

side. In only one of the 17 rats were control-like numbers of PV-ir cells observed in conjunction with a complete abolishment of the 50-kHz USVs after surgery (381.12). And it was precisely in this rat that an unusual pattern of microglial staining was observed: it was the only animal – apart from the two with bilaterally successful lesions (Fig. 3B) – in which activated microglia were located within the region of the paraflocculus nucleus itself. In less successful lesions, they were usually encountered at some distance therefrom. One explanation for the finding might be that in this rat, kainic acid led to death of not the PV core neurons, but potentially of the Foxb1-expressing neurons surrounding them. One limitation of the present study was that there was no possibility to assess the effect of the excitotoxin lesion on the Foxb1 sub-population. Using a strain of mice that expresses Cre recombinase under the control of the Foxb1-promoter [68], it has been possible to characterise in detail the topography and the morphological features of the Foxb1-expressing cells in the paraflocculus nucleus [4]. To date, all attempts to map Foxb1-immunoreactivity in wild-type mice or rats using specific antibodies have failed.

Notwithstanding this limitation, when the raw data pertaining to the individual 17 rats were considered as a whole, irrespective of the group affiliations, a close correlation was found between the total absolute number of PV-ir cells counted on both sides and the surgery-associated change in the number of 50-kHz calls. This correlation was best described by a specific power function (Fig. 8A), which suggests that the elimination of, for example, the first 10% of the PV-ir neurons has a much greater impact on the reduction in the 50-kHz call number than does the elimination of the last 10%.

Not only the surgery-associated reduction in the number of 50-kHz calls that were emitted during the tickling sessions, but

also the surgery-associated reduction in the other two parameters assessed, namely, HAL-positivity index (Fig. 6) and the total hand-following time during the “hand-chase” tickling manipulation (Fig. 7), were most pronounced in the five rats with bilaterally successful lesions. This finding supports our hypothesis that the reduction in the number of 50-kHz USVs observed after successful lesioning of the paraflocculus nucleus is attributable to a change in emotional rather than in motor processing. Averaged across groups, values for both HAL-positivity index and total hand-following time were somewhat reduced immediately after surgery in all other three groups as well. This finding suggests that either the anaesthesia or the surgical manipulations themselves had a negative impact on the tendency of the rats to approach and follow the experimenter’s hand, or that the animals lost interest in or excitement about these tests when they were too often repeated (no research group has before repeated the HAL-test more than four times, and the only effect of repetition reported in the literature is a decrease in approach latency [53,69]). Since in the present study, the group means usually continued to increase even between baseline days 4 and 6, an unspecific effect of the surgical manipulations would appear to be the more likely explanation. However, also for the two parameters used to assess positive affect-related behaviour, close correlations were found between the total absolute number of PV-ir cells counted on both sides and the surgery-associated change in either of these (Fig. 8B & C). The fact that additional positive correlations were found between pairs of the three parameters measured (surgery-associated change in the number of 50-kHz USVs, in the HAL-positivity index and in the hand-following time; Fig. 8D–F), further demonstrates that lesioning affected not only the vocalisations but rather the expression of positive emotions in response

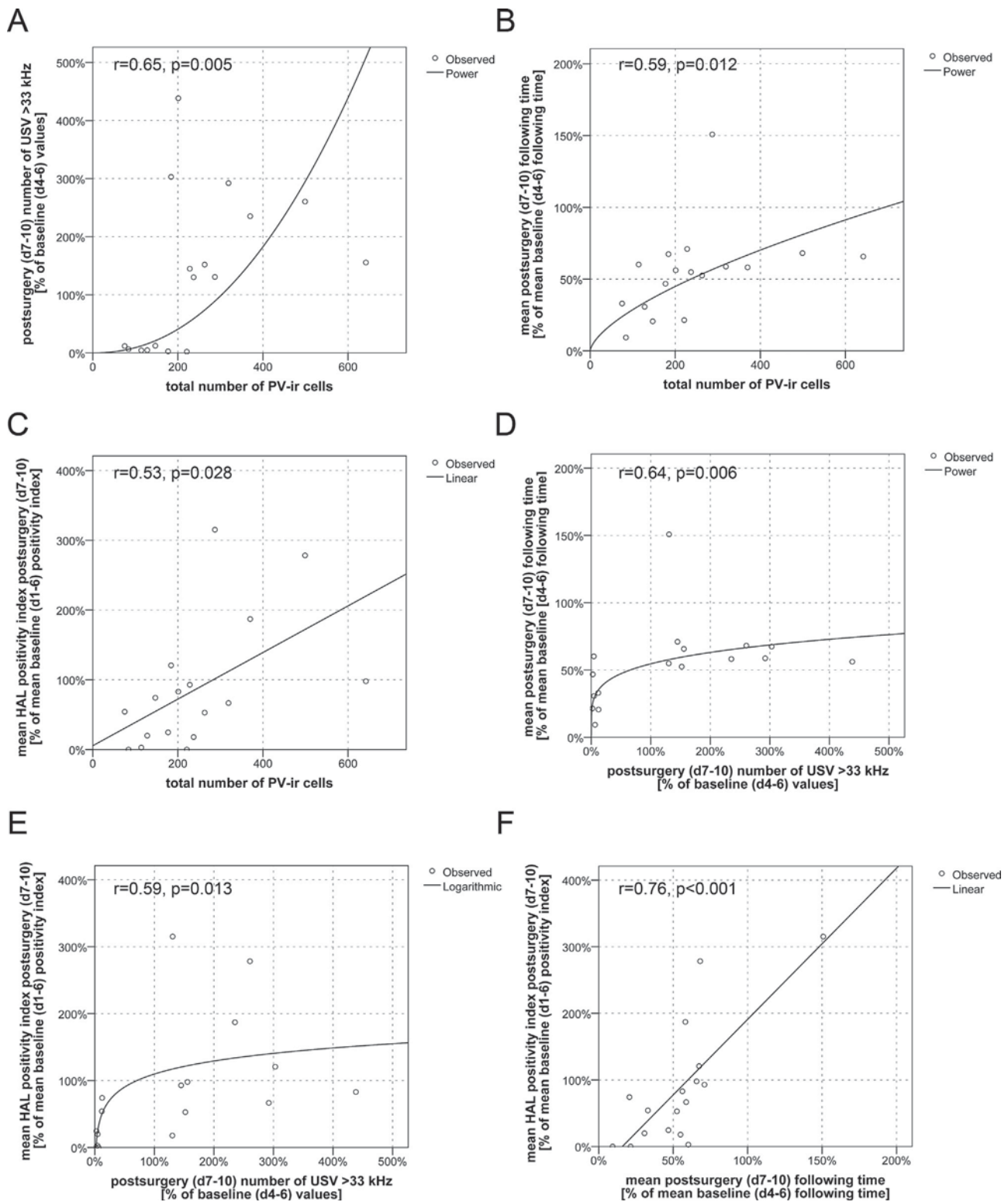


Fig. 8. The number of PV-ir cells correlates with surgery-associated changes in all parameters that were used to assess positive affect-related behaviour. Regression analysis was conducted to probe the nature of the relationship that existed between the various parameters used in the present study to assess positive affect-related behaviour, and between each of these and the number of PV-ir cells. In each graph, the 17 circles represent data pertaining to the 17 individual rats; the continuous line is a trace of the function (amongst the options: linear/logarithmic/power/logistic/exponential) that best fitted the observed points. The relationship between the number of PV-ir cells and the surgery-associated change in high-frequency vocalisations was best described by a power function (A). The relationship between the number of PV-ir cells and the surgery-associated change in the hand-following time was likewise best described by a power function (B). The relationship between the number of PV-ir cells and the surgery-associated change in the HAL-positivity index (HAL-test) was best described by a linear function (C). (D-F): A correlation was found between each of the three parameters used to assess positive affect-related behaviour. The relationship between the surgery-associated change in high-frequency vocalisations and in hand-following time was again best described by a power function (D). The relationship between the surgery-associated change in high-frequency vocalisations and in HAL-positivity index was best described by a logarithmic function (E). Of all the correlations, the linear relationship that existed between the surgery-associated change in hand-following time and in HAL-positivity index was the closest (F). See Section 3 for details of the experimental days included in the calculations for the various parameters.

to the tickling manipulation and the experimenter's hand in its entirety.

We suggest that neurons in the lateral hypothalamus or rather, more specifically, the PV-expressing sub-population of the parvafox nucleus, are involved in the production of 50-kHz USVs. In the context of lateral hypothalamic manipulations, it is of particular importance to distinguish between effects mediated by local neurons and those mediated by by-passing fibres, owing to the presence of the medial forebrain bundle (MFB), which carries fibres of the mesolimbic dopamine system. The lateral hypothalamus – as well as the prefrontal cortex – are counted amongst the brain areas that support the most vigorous self-stimulation behaviour in rats; upon electrical stimulation, they induce 50-kHz calling [22]. In anticipation of a rewarding electrical brain stimulation of the LHA, rats augment the emission of 50-kHz USVs [70]. Obviously, this response is (as least partially) attributable to a stimulation of fibres in the mesolimbic dopamine system running within the MFB, and which is known to increase 50-kHz call-rates [22,70]. However, there is evidence for reward mechanisms originating in the LHA itself, and intracranial self-stimulation in this area was found to be at least partially sustained by an activation not of the passing dopaminergic fibres in the MFB [71], but of local neurons [72,73]. Moreover, the injection of an excitotoxin such as kainic acid into the brain is classically believed to destroy local cell bodies not by-passing fibres. An injection of one of two other excitotoxins, namely ibotenic acid and NMDA, into the lateral hypothalamus has been shown to produce a zone of neuronal killing, that exceeds in extent that of demyelination [74]. The author of the study reasoned that kainic acid might exert a less severe demyelinating effect than either ibotenic acid or NMDA. In other studies, an injection of either kainic acid [75] or ibotenic acid [76] into the LHA induced the degeneration of neuronal bodies but no significant destruction of by-passing fibres (of the MFB).

Kainic acid acts as an agonist on glutamate receptors of both the kainate and the AMPA subtypes [77]. Although kainate receptors along axons are possible, these are usually located close to active zones [78]. Kainic acid-induced neuronal death in the rat hippocampus is accompanied by an increase in the proliferative rates of astrocytes and microglia [79], but rather not by a complete destruction of by-passing axons. Since the volumes of kainic acid injected in the present study did not suffice even to eliminate in entirety the small PV sub-population of parvafox neurons, it is reasonable to assume that the excitotoxin did not interfere with the function of the mesolimbic dopamine system by directly destroying by-passing fibres.

Any local neuron in the region of the parvafox nucleus could of course have been affected by the lesions and have contributed to the observed post-surgical phenotype. However, the correlations found between the number of PV-ir parvafox cells and the surgery-associated change in all three of the investigated parameters (number of 50-kHz USVs, hand-following time, HAL-positivity index) suggest a significant contribution of this sub-population.

Broadening our view beyond the rat species, evidence for a role of PV-expressing neurons in the context of vocalisation already exists. PV-expression in the songbird is confined to specific neuronal populations that are especially involved in song-learning [80–82]. This finding suggests that PV fulfills a specific function in these neurons that is potentially related to their morphological plasticity in the context of vocal learning (see also [83]); or, alternatively, to their protection against Ca^{2+} -toxicity from the highly active neurons that control some of the fastest muscles in the body [84]. Furthermore, in avian as well as in primate species, PV is differentially up-regulated in the vocal motor neurons of vocal learners (learned songs or spoken language) compared to non-learners [85]. And in PV-knockout mice, deficits in social interaction as well as in communication are observed [86]. Hence, the neuronal

properties that are mediated by the Ca^{2+} -buffer PV might be particularly required by neurons involved in the production of vocalisations.

The precise role played by the parvafox nucleus in the production of vocalisations, as well as in the context of positive emotions, remains elusive. Bearing in mind the complex and wide-ranging connections of the two sub-populations of neurons that comprise the parvafox nucleus, namely, the PV-ir core cells and the Foxb1-expressing ones of the shell surrounding this core, as well as the partially opposing functions of the brain centres targeted by them, we hypothesise that the functions are not confined to the production of vocalisations, but embrace also on the generation or display of specific emotional states, which possibly includes an interaction with various manifestations of the autonomic nervous system [87,88] and perhaps also with nociception pathways [89–91].

In summary, the present study affords evidence that the parvalbumin expressing sub-population of neurons in the hypothalamic parvafox nucleus plays a role in the expression of positive emotions in rats as manifested by the emission of ultrasonic vocalisations and animal approach behaviours.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbr.2015.11.004>.

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