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Electrical activity in rat cortico-limbic structures after single or repeated administration of lipopolysaccharide or staphylococcal enterotoxin B

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Immune-to-brain communication is essential for an individual to aptly respond to challenging internal and external environments. However, the specificity by which the central nervous system detects or 'senses' peripheral immune challenges is still poorly understood. In contrast to post-mortem c-Fos mapping, we recorded neural activity *in vivo* in two specific cortico-limbic regions relevant for processing visceral inputs and associating it with other sensory signalling, the amygdala (Am) and the insular cortex (IC). Adult rats were implanted with deep-brain monopolar electrodes and electrical activity was monitored unilaterally before and after administration of two different immunogens, the T-cell-independent antigen lipopolysaccharide (LPS) or the T-cell-dependent antigen staphylococcal enterotoxin B (SEB). In addition, the neural activity of the same individuals was analysed after single as well as repeated antigen administration, the latter inducing attenuation of the immune response. Body temperature and circulating cytokine levels confirmed the biological activity of the antigens and the success of immunization and desensitization protocols. More importantly, the present data demonstrate that neural activity of the Am and IC is not only specific for the type of immune challenge (LPS versus SEB) but seems to be also sensitive to the different immune state (naive versus desensitization). This indicates that the forebrain expresses specific patterns of electrical activity related to the type of peripheral immune activation as well as to the intensity of the stimulation, substantiating associative learning paradigms employing antigens as unconditioned stimuli. Overall, our data support the view of an intensive immune-to-brain communication, which may have evolved to achieve the complex energetic balance necessary for mounting effective immunity and improved individual adaptability by cognitive functions.

Keywords: telemetry; cytokines; insular cortex; amygdala; lymphocytes; tolerance

1. INTRODUCTION

The cornerstone of the immune-to-brain interactions was the observation in rats that days after the initial inoculation of sheep erythrocytes, when the primary immune response peaks, increased firing rates were recorded in the hypothalamic ventromedial nucleus [1]. This discovery initiated the conceptualization of lymphocytes sharing a common chemical language with neurons for intra- and inter-system communication [2]. Surprisingly, however, after some attempts [3–8], only minor progress has been achieved in understanding the neural processing and encoding of lymphocyte signalling. Another provoking finding was the possibility to induce a behaviourally conditioned immune response employing antigens or immunomodulatory drugs as unconditioned stimuli [9]. In overall, these data raised the hypothesis that the brain has the capacity to recognize, encode and store signalling from the immune system.

Clinical and experimental evidence support the physiological relevance of immune-to-brain communication [10–13], and several underlying pathways have been well identified [14,15]. Vagal afferences were initially implicated as the route by which immune signals access the brain [16,17]; however, opposing data challenge the vagus monopoly, brain-resident macrophages and endothelial cells being essential players on the lymphocyte-to-neuron transduction processes [18–20]. Cytokines are key intercellular messengers orchestrating immune responses, but also have direct and specific neuronal effects [21,22]. They activate discrete networks within the hindbrain-, hypothalamic- and cortico-limbic structures [23,24], consequently affecting behaviour such as lethargy, depression, anorexia, sleepiness and hyperalgesia, collectively termed as 'sickness behaviour' [14].

Lipopolysaccharide (LPS) and staphylococcal enterotoxin B (SEB) are bacterial products, both eliciting fast, robust but distinct immune responses. LPS mainly

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triggers $CD14^+$ myeloid cells, without the initial involvement of T lymphocytes (i.e. T-cell-independent antigen), leading to a particular profile of pro-inflammatory cytokine release (e.g. IL-1β, IL-6 and TNFα) [25]. In contrast, the superantigen SEB strongly activates T lymphocyte clones via the T-cell receptor (V-β8), concomitantly resulting in increases of IL-2 and other cytokines in the blood [26,27]. The magnitude of the immune response to both antigens largely depends on the individual immune history; in contrast to the robust immune response during the first antigen encounter with pronounced cytokine release, repeated inoculations of LPS or SEB lead to desensitization, significantly dampening peripheral immune responses such as cytokine production [28,29]. Peripheral insults activate lymphocytes, leading to local and self-regulated processes, but also are strongly modulated by autonomic regulatory loops [30–32]. Importantly, the levels of circulating cytokines parallel the capacity of LPS to be used as effective unconditioned stimulus in associative learning paradigms [33,34], indicating the relevance of pro-inflammatory cytokines in the immune-to-brain signalling process.

Post-mortem c-Fos mapping, with poor time resolution, has been the choice for documenting a neural network responsive to peripheral immune challenges. So far, this network includes: the nucleus of the solitary tract, area postrema, parabrachial nucleus in the hindbrain, central nucleus of the amygdala (Am) and hypothalamic paraventricular nucleus in the forebrain [23,35–40]. However, longitudinal studies on neuronal responses after one or repeated antigen inoculation are completely lacking. Therefore, in order to analyse how and to what extent two specific immunogens commonly employed as unconditioned stimuli (LPS versus SEB) affect relevant cortico-limbic structures activity, we recorded electrical activity of the Am and in the insular cortex (IC) during the first 200 min after the first or repeated antigen administrations, respectively. We employed a wireless recording method, allowing unrestricted animal behaviour, minimizing stress artefacts and providing in our hands reliable electrophysiological data [41]. The study focused on the Am and IC since these structures have previously been shown to be involved in immune-visceral inputs, independently of the afferent route involved [36,42–44], and because lesion experiments documented the relevance of these two structures during processing immune signals employed as unconditioned stimuli [45–47]. Furthermore, in independent experiments, changes in body temperature and circulating cytokine profiles were monitored to further evaluate the immune reactivity under the various experimental conditions.

2. MATERIAL AND METHODS

(a) *Animals*

Male Dark Agouti rats (250–300 g, Harlan, Netherlands) were individually housed under a normal 12 L : 12 D schedule (lights on at 7.00 h) with food and water available ad libitum.

(b) *Antigens*

Two different antigens were selected: LPS from *Escherichia coli* (serotype O55 : B5, Sigma, Germany) and *Staphylococcus* enterotoxin B (SEB, Sigma). Both antigens were diluted in sterile saline and applied intraperitoneally (i.p.).

Table 1. Study design and variables analysed.

	naive				desensitization
experimental day	1	2	3	4	5
Veh (saline 1 ml)	X	X	X	X	X
LPS (mg kg^{-1})	0.1	0.2	0.5	0.5	0.5
SEB (mg kg^{-1})	1.0	1.0	1.0	1.0	1.0
neural recordings	X				X
temperature	X	X	X	X	X
cytokines	X				X

(c) *Study design*

Treatment regimen and study design are summarized in table 1. Three independent experiments under identical conditions were performed for acquiring data on (A) neural recordings, (B) cytokines, and (C) body temperature. Immune desensitization was induced as previously described for LPS [48] and SEB [29]. Briefly, rats received i.p. injections of either ascending doses of LPS (0.1, 0.2, 0.5, 0.5 and 0.5 mg kg^{-1} b.w.) or constant doses of SEB (1.0 mg kg^{-1} b.w.) over a period of 5 consecutive days, whereas controls were injected with vehicle (1 ml sterile saline). Antigen doses and application intervals were selected based on previous experience in our laboratory [33,34]. After the first and fifth application, neuronal activity (Exp A), cytokine levels (Exp B) and body temperature (Exp C) were analysed.

(d) *Neural recordings*

Two independent experiments were performed, one for LPS ($n = 8$) + vehicle ($n = 6$) and other for SEB ($n = 10$) + vehicle ($n = 7$). Deep-brain electrode implantation and neural recordings were performed as described previously [41,49]. Briefly, under deep anaesthesia with xylazine/ketamine i.p. (5 mg kg^{-1}/90 mg kg^{-1}), monopolar stainless steel electrodes were unilaterally implanted into the Am and the ipsilateral IC at coordinates relative to the Bregma (Am: posterior −2.5 mm, lateral 4.3 mm, ventral 8.0 mm; IC: anterior +1.6 mm, lateral 4.8 mm, ventral 6.5 mm). Coordinates were selected based on previous lesion experiments [45], corresponding to the anterior IC and the caudal Am, responsible for visceral processing [42,44,50]. Lateralization was controlled by equally distributing left and right hemispheres across the groups. A stainless steel screw used as an indifferent electrode was positioned on the skull at the surface of the cerebellum. Electrodes were soldered to a socket (TSE-Systems, Bad Homburg, Germany) and fixed with dental cement on the skull. After surgery, re-hydration, analgesics and prophylactic antibiotics were applied. Before any experimental treatment, animals were allowed to recover for a period of at least 14 days, time enough to metabolize peri-operative drugs and to reduce blood–brain barrier leakage. To monitor the neural activity of freely moving rats, a calibrated transmitter (TSE Systems) was fixed on the electrode socket by means of a plug connection. The neural signals were telemetrically received via pulse position modulation and transmitted to a decoder. Data acquisition was coupled online to a computer that transformed the data into real time by means of fast Fourier transformation analysis and displayed them as absolute power (μV) as well as

continuous spectra of power density. The mean absolute power was calculated for the total power, summarizing the power of the whole frequency range (0.6–30 Hz). Sampling rate of the digitized neural signals was 128 s^{-1}. By coupling the data acquisition to a SIGMA PLpro EEG device with simultaneous video recording of the behaviour of the rat (Neurowerk, Gelenau, Germany), raw recordings were visualized on the computer screen and inspected to check for the presence of any anomalous patterns. The baseline was composed of three records (5 min every 15 min, 1 h before treatment). After antigen injection, recordings started again for 5 min every 15 min (first time point 20 min post-immune challenge). Data were analysed as power index (total power at a given time point/baseline total power) to account for the changes over time. To compute the magnitude of change, an integrated power index was calculated as the area under the curve (Sigma Plot) during the whole recording period (20–200 min).

(e) *Cytokine determination*

To analyse plasma cytokine concentrations, blood was collected sublingually under light inhalation anaesthesia (Isoflorane) as described previously [33], 2 h after antigen injections, LPS ($n = 6$) and SEB ($n = 6$). Time point was selected based on previous experience with both antigens [34,48]. Plasma was collected after centrifugation (5 min, 10 000 rpm, 4°C) and stored at −80°C until cytokine determination. To obtain splenocytes, animals ($n = 10$) were sacrificed 2 h after the last injection and spleens were collected. Single-cell suspensions of the spleen were obtained as described previously [51]. Briefly, after mechanically disrupting the tissue and erythrolysis, splenocytes were washed, filtered and adjusted to a concentration of 2.5×10^6 cells ml^{-1} and stimulated in 96-well flat-bottom microtitre plates with 1 μg ml^{-1} of anti-rat CD3 monoclonal antibody (clone: G4.18; BD Pharmingen) or SEB (10 μg ml^{-1}) for 72 h in a humidified incubator (37°C, 5% CO_2).

Employing bead-based multiplex assays, cytokine levels in plasma or culture supernatants were quantified as described previously [51]. Briefly, sample dilutions were incubated with fluorescence-labelled beads that are coupled to monoclonal antibodies against rat IL-1β, IL-2, IL-6, TNFα and IFN-γ. Upon incubation with cytokine-specific detection antibodies, samples were incubated with streptavidin-PE (Becton Dickinson). Per sample, 200 beads were analysed on a dual-laser flow cytometer. Absolute cytokine levels were calculated based on the mean fluorescence intensity of cytokine standards. The detection limits of the assays were 0.1 pg ml^{-1} for IL-1β, IL-2 and IL-6, 0.5 pg ml^{-1} for IFN-γ and 1.4 pg ml^{-1} for TNFα, respectively. To account for inter-individual variability, cytokine levels after the fifth injection were standardized as the intra-individual percentage of the first day.

(f) *Body temperature*

Body temperature was continuously recorded via implanted data-loggers (mini-Subcue Dataloggers, Calgary, Canada), as described previously [52]. Briefly, data-loggers were surgically i.p. implanted ($n = 17$) two weeks before starting the experiment and were set to record every 15 min. At the end of the experiment, the data-loggers were explanted, temperature data downloaded and corrected. Temperature data were integrated as the area under the curve (SigmaPlot) between 160 and 630 min post-challenge, expressed as fever index [53] for day 1 and day 5.

(g) *Statistical analysis*

Data were analysed using SPSS (v. 17.0) for parametric assumptions. When necessary, robust statistic transformations were employed as indicated. Relevant and appropriate tests were employed for each dataset with a common level of significance set at $p < 0.05$: two-way mixed ANOVA for body temperature; one sample *t*-test for plasma cytokines; one-way ANOVA for supernatant cytokines; mixed ANOVA and *t*-tests for neural recordings.

3. RESULTS

(a) *Body temperature*

LPS and SEB pyrogenicity are well documented and established in our laboratory [52]. Thus, in a first experiment, we analysed body temperature before and after peripheral administration of LPS ($n = 5$), SEB ($n = 6$) or Veh ($n = 6$) after one (naive condition) as well as after repeated antigen challenge (desensitization). Fever was clearly elicited in animals receiving the first LPS or SEB injection (electronic supplementary material, figure S1). Two-way mixed ANOVA, with treatment (Veh, LPS and SEB) and day (first and fifth) as factors yield significant $p < 0.05$ day effect $F_{1,14} = 21.638$ and day × treatment interaction $F_{2,14} = 4.122$. These data document the pyrogenic capacities of LPS and SEB under naive conditions and clearly show that after repeated administration, the pyrogenicity of the same antigen is significantly attenuated.

(b) *Plasma cytokines*

We measured the peripheral cytokine response after single and repeated injections of LPS ($n = 6$) and SEB ($n = 6$), respectively. Repeated injections of either LPS or SEB induced a significant attenuation of the cytokine response. For LPS, the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β were significantly decreased after desensitization compared with the levels elicited under the naive condition, confirming our previous reports [33,48]. Simple *t*-tests indicate that all three cytokines were significantly blunted after the fifth injection of LPS: TNFα, IL-6 and IL-1β (electronic supplementary material, figure S2*a*, left). Similarly, we could document an *in vivo* SEB desensitization by means of the blunted production of IL-2 and IL-1β (electronic supplementary material, figure S2*a*, right). Superantigens, like SEB, induce a strong proliferative response followed by clonal deletion of a substantial portion of defined Vβ T cells [27], and it has been documented that the remaining cells display an *in vitro* anergy status [26]. Thus, we tested this status of non-responding to the antigen in an *in vivo* approach. Isolated splenocytes from animals repeatedly injected with either vehicle ($n = 5$) or SEB ($n = 5$) (5×1 mg kg^{-1}) were challenged *in vitro* with either anti-CD3 or again with SEB (10 μg ml^{-1}). The robust IL-2 response induced by vehicle *in vivo*/SEB *in vitro* challenge was significantly blunted in the SEB *in vivo*/SEB *in vitro* condition, as indicated by the Games–Howell post hoc test after one-way ANOVA, $F_{3,7.57,\text{Welch}} = 13.70$ (electronic supplementary material, figure S2*b*). This effect was specific for SEB reactive

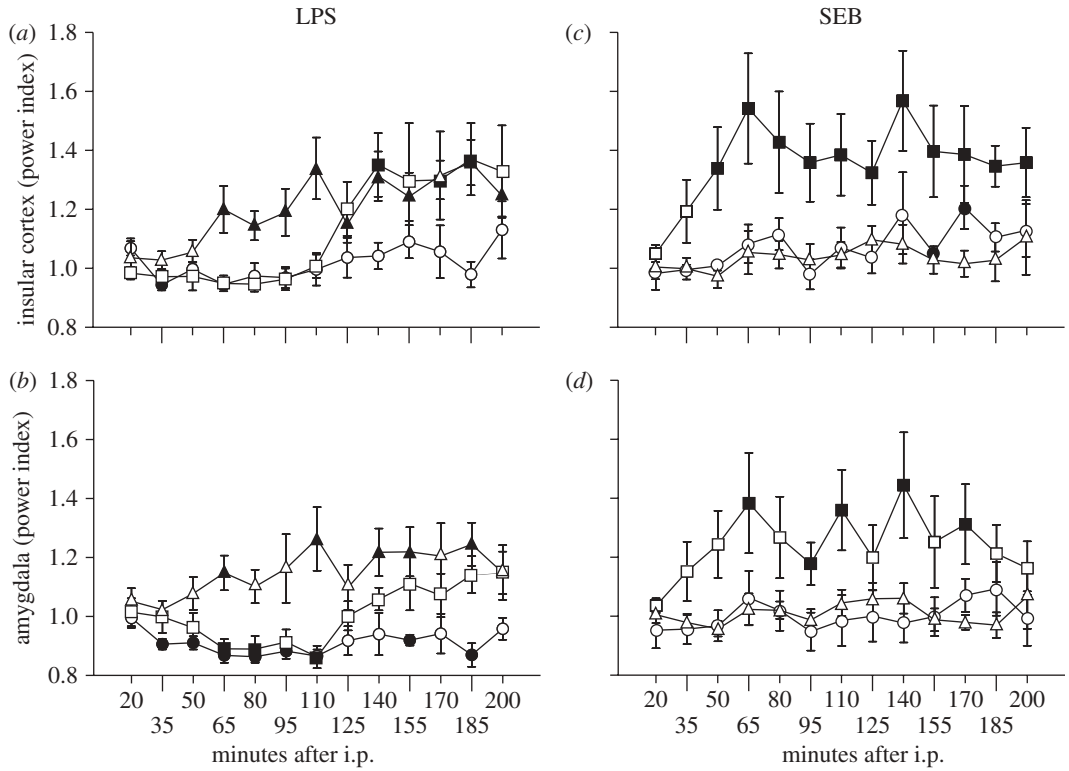

Figure 1. Cortico-limbic dynamics during early phase of the immune response. Insular cortex and amygdala activity were followed with a wireless method; monopolar deep-brain electrodes coupled to telemetry system. The baseline was composed of three recordings (5 min every 15 min, 1 h before treatment). After treatment (first day: Veh $n = 13$, LPS $n = 8$, SEB $n = 10$; fifth day: LPS $n = 8$, SEB $n = 10$), recordings started again for 5 min every 15 min with the first time point 20 min post-challenge. Data expressed as power index (time point total power/baseline total power) over time. Challenge under naive = first injection (squares), or after desensitization = fifth injection (triangles). As negative control, vehicle (circles) was administered. Closed symbols depict $p < 0.05$ versus baseline, one-sample *t*-test.

Table 2. Baseline neural activity (total power) in the left and right hemispheres before any i.p. injection. Units: microvolts, mean ± s.e.m. Data include a total of 49 recordings, including data collected on the first ($n = 31$) and fifth day ($n = 18$).

insular cortex		amygdala	
left ($n = 24$)	right ($n = 25$)	left ($n = 24$)	right ($n = 25$)
5314 ± 244	4811 ± 199	6469 ± 288	5800 ± 313

clones, since anti-CD3 *in vitro* challenge was able to elicit a comparable IL-2 production regardless of the *in vivo* challenge. IFN-γ levels were also significantly affected by SEB *in vivo* challenge when tested *in vitro* with either anti-CD3 or SEB, as indicated by the Bonferroni post hoc test after one-way ANOVA, $F_{3,16} = 245.29$ (electronic supplementary material, figure S2*b*). These data confirmed a desensitization of the immune response after repeated exposures to LPS and SEB.

(c) *Neural activity*

We analysed the neural activity in the IC and Am via radio-telemetry in the following groups of animals: vehicle $n = 13$, LPS $n = 8$, SEB $n = 10$. Recordings of the baseline are summarized in table 2, corresponding to 31 ($n = 13$ vehicle/$n = 8$ LPS/$n = 10$ SEB) recordings to the first day and 18 to the fifth day ($n = 8$ LPS/$n = 10$ SEB). There was no significant difference between left and right hemispheres for IC, $t_{47} = 1.605$, as well as for Am, $t_{47} = 1.567$. Therefore, data of both hemispheres were pooled for each brain structure for further analysis. Under naive condition (first injection), LPS significantly affected neural activity in the IC and Am (figure 1*a*,*b*). The IC positively responded to a 0.1 mg kg^{-1} LPS challenge with a latency of 140 min, peaking at 185 min (table 3). When integrated, the magnitude of change was significantly different from vehicle, $t_{13} = -2.403$, $p < 0.05$ (figure 2*a*). The Am also responded to LPS around 110 min after inoculation (figure 1*b*). When integrated, the magnitude of change was significantly higher than the integrated power index of vehicle (figure 2*b*). Regarding SEB, the IC reacts faster, with a latency of 50 min (figure 1*c*, table 3). The magnitude of change was the largest of all of the conditions tested, being significantly different from the vehicle response $t_{13} = -3.777$, $p < 0.05$. For this antigen, the Am mostly parallels the activity of the IC, with a latency of 65 min and sustained increments (figure 1*d*, table 3), and a significant magnitude of response (figure 2*d*) $t_9 = 3.889$, $p < 0.05$.

Five days later, after the same subjects were submitted to repeated injections of the same antigen (either LPS or SEB), their neural activity was again monitored and analysed for dynamics and magnitude changes. Regarding LPS, the latency in the IC was shorter (65 min) than in the naive condition (figure 1*a*, table 3). However, the

Table 3. Latency and peak of insular cortex and amygdala activity after immune challenge. Data are expressed as minutes; latency, first significant change from baseline; peak, maximal deviation from baseline; (+) or (−) signs indicate the direction of change in comparison to baseline; n.s., not significant.

	naive (day 1)				desensitization (day 5)			
	insular cortex		amygdala		insular cortex		amygdala	
	latency	peak	latency	peak	latency	peak	latency	peak
LPS	140 (+)	185 (+)	65 (−)	n.s.	65 (+)	140 (+)	65 (+)	110 (+)
SEB	50 (+)	140 (+)	65 (+)	140 (+)	n.s.	n.s.	n.s.	n.s.

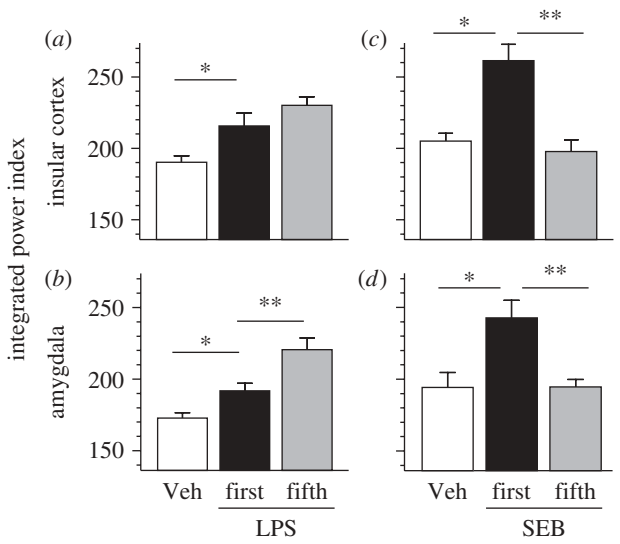

Figure 2. Cortico-limbic magnitude during early immune response (first day: Veh $n = 6 + 7$, LPS $n = 8$, SEB $n = 10$; fifth day: LPS $n = 8$, SEB $n = 10$). Data expressed as integrated power index (area under the curve 20–200 min post-injection; trapezoid method). Challenge under naive = first injection (filled bars), or after desensitization = fifth injection (grey bars). As negative control, vehicle (open bars) was administered. *$p < 0.05$ first versus vehicle (Veh), independent *t*-test; **$p < 0.05$ first versus fifth injection, dependent *t*-test.

total magnitude was not affected (figure 2*a*). As on the first day, the Am followed a similar dynamic (table 3 and figure 1*b*); however, in this structure, the magnitude was significantly higher compared with the first encounter with LPS (figure 2*b*), $t_7 = -2.857$, $p < 0.05$. Importantly, repeated injections of SEB evoked a response completely opposite to LPS, resulting in blunted neural activity (dynamics/magnitude), being even now similar to the vehicle condition (table 3 and figures 1 and 2*c*,*d*).

4. DISCUSSION

Anatomical and functional evidence accumulated during the last decades document a close interaction of the immune and nervous systems on homeostasis maintenance [10,12]. Our data extend the view of the specificity of immune-to-brain communication during the early minutes of the immune response. Already within the first 200 min after antigen inoculation, the dynamics and magnitude of the electrical activity recorded from the IC and Am were different depending on the antigen administered: T-cell-dependent (SEB) versus T-cell-independent (LPS) antigen. Moreover, in the same individual, we documented that the immune history significantly affects the neural response to the same antigen challenge; i.e. after desensitization, both antigens induced a different neural response, indicating a different perception of the same antigenic challenge. Furthermore, these data provide the electrophysiological substrate for associative learning paradigms employing these antigens as unconditioned stimuli. In summary, our data highlight cortico-limbic dynamics and specificity related to the peripheral immune reactivity within its very early stages.

Hypothalamic nuclei were initially screened and found to be reactive during the effector phase of the humoral immune response (days after inoculation) in anaesthetized [1] and in conscious rats [6,7,54,55]. We took advantage of two stereotypic bacterial antigens that elicit fast and well-characterized immune responses [25,56]. Additionally, these antigens induce corticosterone release [34,57–59], considered to be part of the adaptive suppressive neuroendocrine response necessary to control immune reactivity [30]. Furthermore, both antigens have been also employed as unconditioned stimuli in associative learning protocols, inducing a conditioned taste avoidance as well as other conditioned responses; fever, sympathetic activity, glucocorticoids and cytokines among other parameters [34,48,60,61]. We further focused on the IC and Am as two structures known to be involved in immune-to-brain communication in general [42,44,62–64], and in the behaviourally conditioned immune response in particular [45–47]. We found no lateralization of the baseline neural activity, and a short latency for the two antigens tested (65–140 min for LPS, 50–65 min for SEB). This time frame parallels pro-inflammatory cytokine production and release into circulation for SEB [59,65] and for LPS [48]. Because neither of the antigens employed cross the blood–brain barrier in appreciable concentrations [66], and deep-brain recordings took place long enough to recover the blood–brain barrier integrity, an alternative afferent route would be of relevance with likely intermediate messengers such as cytokines or prostaglandins [17,39,67–70]. In the case of LPS, substantial evidence indicates that circulating cytokines can be monitored by non-neuronal cells of the cerebral vasculature, thereupon releasing prostaglandins to reach neurons, independently of vagal afferences [39,69,71]. In contrast, disruptions of interoceptive signalling by area postrema lesions or vagotomy markedly attenuated SEB- but not

LPS-stimulated brain c-Fos expression [24,39] indicating that LPS and SEB appear to use distinct mechanisms to signal the brain. In addition to the different afferent routes employed, our data document a distinct neural processing of these two stereotypic antigens; the dynamics and magnitude of cortical and amygdaloidal activity differ during the particular immune responses elicited, confirming the proposed specificity of the immune signalling processes [23,72]. Furthermore, this is the first longitudinal *in vivo* evidence documenting how rapidly the brain reacts to peripheral immune challenges, supported on many other post-mortem metabolic mapping reports [24,35,39,40]. In this regard, our recordings on the IC are the first to indicate a clear involvement of this neocortical area in the early processing of peripheral immune inputs, which was not previously allocated by c-Fos expression. However, it should be mentioned that the IC has been widely involved in processing visceral inputs [62,63] as well as in associating immune stimuli with other exteroceptive stimulation within the acquisition phase of Pavlovian conditioning [45–47]. Substantial evidence supports the Am as relaying visceral inputs [64,73] in addition to its well-known function in arousal [74]. Our electrophysiological recordings from the Am are in agreement with data from chinchilla rabbits following lateral Am activity after immunization with different antigens (e.g. sheep erythrocytes, rat erythrocytes and horse globulin) [4,75], confirming a prominent role of this limbic structure within the processing of signals released during the early phase of an immune response [36]. Electrophysiological recordings, while offering *in vivo* neural activity assessments and longitudinal experimental designs, limit the number of regions to be analysed in comparison to post-mortem metabolic mapping. In this regard, it is well known that severe ablations of the IC and the Am might interfere with peripheral immune reactivity [76,77]. However, our controls have been treated in the same way; thus, comparisons are valid but might differ from completely intact rats. Another potential confounder is the stress response induced by the electrophysiological recording apparatus. In this regard, we took advantage of wireless technology allowed by radio-telemetry to acquire electrical neural activity in completely free-behaving rats [49,78,79], which might reduce stress artefacts.

Distinct neuro-behavioural responses have been documented when comparing the same individual under antigen-naive versus antigen-experienced conditions [33,80,81]. We have reported that LPS alienates its capacity as an unconditioned stimulus in endotoxin-tolerant animals, which might be due to the absence of pro-inflammatory cytokines [33]. Against this background, we tested the hypothesis that differences in the immune response would be also reflected in neural activity of the IC and Am. The blunted cytokine levels and attenuated fever response confirm desensitization after repeated injections of either SEB or LPS, supported by previous reports [29,48,53,80]. Importantly, corticolimbic activity also was modified by the immune desensitization occurring in the periphery. For SEB, both IC and Am neural activity were significantly reduced after repeated inoculations, supporting the blunted cytokine response in the periphery after repeated applications. Our data support desensitization effects previously reported for repeated and frequent inoculations of this superantigen [28,29]; however, it should be mentioned that not all cytokines were blunted. Interestingly, for the staphylococcal enterotoxin A, the interval between immunizations may be an important factor of the magnitude of desensitization or even sensitization effects [82]. The immunological mechanisms underlying such effects remain elusive, but are likely to be dependent on the kinetics of the T-cell proliferative response after single versus repeated and frequent superantigen injections.

However, for LPS, we observed a paradoxical response, since the IC latency was shortened and the magnitude of the neural response was similar to that observed when LPS was administered in naive immune condition. In this regard, Am activity also showed increased electrical activity after repeated LPS injections when circulating cytokines were blunted, additionally demonstrating significantly higher activity than under naive condition. Potential explanations for this phenomenon are: (i) the used LPS regimen implies increasing doses, starting with $0.1\ \mathrm{mg\,kg^{-1}}$ on day 1 reaching $0.5\ \mathrm{mg\,kg^{-1}}$ on day 5, which may lead to a potentially confounding dose factor, (ii) other messengers than cytokines may be involved in the afferent communication on day 5, for instance, prostaglandins released by endothelial and/or perivascular cells as a response to circulating LPS [20,39], and (iii) plasticity phenomena leading to neuroimmune sensitization [83] or associative learning [48]. Finally, whether and to what extent the observed changes are 'immune specific' can only be provided in another set of experiments, in which the activity of immunocompetent cells and/or peripheral cytokines will be abolished with concurrent neural recordings. Supporting this argument, we have reported that when using a protein antigen as immune challenge, brain c-Fos expression is completely blunted by a general immunosuppressive pretreatment [38]. Furthermore, amygdaloid c-Fos mRNA is also blunted after repeated LPS administrations [84].

The immune → neuro → immune loop is essential for energy homeostasis [85]. Immunity involves a substantial energy investment [86]; therefore, lymphocytic activity is subject to a trade-off between other energy-demanding processes, such as reproduction, foraging/hunting, social interaction, resulting at the end in the so-called 'sickness syndrome' [14]. On the afferent arm, as mobile sentinel, immune cells are well positioned to constantly survey the organism and to detect pathogenic insults (either self or non-self) responding to it, but simultaneously via immunotransmitters (cytokines, prostaglandins, or neurotransmitters) informing the brain by means of neural and humoral afferent pathways [15]. Theoretically, this immune-to-brain interaction may also codify stimulus modalities (intensity, location and duration). Our data comparing two stereotypic immunogens, LPS versus SEB, substantiate the view of an immunoception process [23], and support the concept of an immunological homunculus [72]. We hypothesize that the sensory capacity to detect relevant immune-borne molecules may lead to the evolutionary advantage of gathering appropriate information for the brain to integrate it with exteroceptive stimuli and/or previous experiences, and to aptly respond, for instance, by allocating fuel storages to energy-demanding cells, i.e. lymphocytes under clonal expansion [85], as well as to learn and anticipate environmental

threat and prepare the body for counteraction by associative learning processes [87].

In summary, we demonstrated in an electrophysiological longitudinal *in vivo* study that two relevant corticolimbic structures, the IC and the Am, are differentially reacting after administration of two different antigens: LPS versus SEB. In addition, the IC and the Am were also sensitive to the individual immune history in naive versus desensitization state. These data support the view of the immune system as a sensory organ, substantiate associative learning paradigms employing antigens as unconditioned stimuli and provide strong evidence of the necessity of moving from post-mortem metabolic mapping to *in vivo* longitudinal studies for a better understand the complex physiology occurring during immune-to-brain interactions.

The experiments were carried out following the current Swiss and German regulations for animal experimentation and were approved by relevant local animal ethics committees.

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